# Identification of liver stem-like cells in human derived intrahepatic biliary epithelial cells *in vitro*

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Dedicated to

my wife

**Yu-lan Xu**

and my parents

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# Identification of liver stem-like cells in human derived intrahepatic biliary epithelial cells *in vitro*

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# 1. INTRODUCTION

## 1.1. Embryology of the liver

The liver of vertebrates originates from an outgowth of the primitive gut wall caudal to the stomach, pancreas and gall bladder, and consists of endodermal and mesodermal components. These give rise to hepatoblasts and both sinusoidal-lining cells and connective tissue, respectively (Shiojiri et al. 1991). The primitive hepatoblasts which surround the portal mesenchyme form a double layered cylinder of hepatocytes, the 'ductal plate', which remodels and migrates into the mesenchyme to form the intrahepatic bile ducts (Shiojiri et al. 1991) (Van Eyken et al. 1988). The hepatoblasts express the hepatocyte-specific protein alpha-fetoprotein (AFP) and albumin as they migrate into the portal stroma and additionally begin to express the bile duct specific marker, gamma glutamyl transferase (γ-GT) (Shiojiri et al. 1991). Initially intermediate filament expression is restricted to cytokeratin (CK) 8, but, during the later stages of ductular morphogenesis, the new ductal cells begin to express the characteristic biliary cytokeratins 7, 8, 18, and 19 (Moll et al. 1982), although continuing to express hepatocyte traits for the first 7-14 days (in rat) after birth (Shiojiri et al. 1991). In contrast, hepatoblasts without contact to the portal mesenchyme differentiate into hepatocytes, which form the liver cell plates and continue to express GGT until birth; the hepatocyte cytokeratin complement is restricted to 8 and 18.

In the human developing liver, the first antigens detected in immature progenitor cells of the liver primordium are CK 8, CK 18, CK 19 and hepatocyte paraffin 1 (HepPar1) antigen at 4 weeks of gestation. Between 8 and 14 weeks' gestation, bipotential progenitor cells appear. They also express CK 14 but are negative for vimentin. Although AFP expression can first be detected at 4 weeks of gestation, at 9-10 weeks of gestation up to 50% of individual hepatoblasts are positive and the number of expressing cells continue to rise. At about this stage, hepatoblasts also express albumin and alpha1-antitrypsin. Between 14 and 16 weeks of gestation, as hepatoblasts differentiate into hepatocytes, CK 14 and CK 19 disappear, whereas hepatocytic markers (HepPar1, albumin, AFP) become more strongly expressed. In contrast, commitment to bile duct cells lineage is marked by increased expression of CK 19 (along with CKs 8 and 18), loss of CK14 and HepPar1 antigen and transient (from 9 –36 weeks) expression of vimentin. Starting from 20 weeks of gestation, bile ducts also show increasing immunoreactivity for CK 7. During the first weeks of biliary development from ductal plate cells (in about 7-9 weeks of gestation), the cells are also positive for AFP, which eventually becomes lost in developing bile duct cells (Haruna et al. 1996).

The liver stem cell is defined as a progenitor cell without distinguishing structure or phenotypic markers that has the capacity to proliferate and produce progeny that may differentiate into either

biliary or hepatic cells (Sell 1990) (Thorgeirsson 1993; Thorgeirsson 1996). Thus, it appears that in the developing livers, hepatoblasts and primitive biliary cells belong to liver stem cells.

### 1.2. Cell types of the postnatal liver

The postnatal liver is composed of parenchymal cells (hepatocytes) and nonparenchymal cells (NPCs). Nonparenchymal cells in the liver include biliary epithelial cells (BECs), kupffer's cells, pit cells, endothelial cells, hepatic stellate cells (HSC, synonyms are Ito cells or fat-storing cells) (Marceau et al. 1989). Hepatocytes comprise about 80% of total cellular population in human liver (60% in rat liver) and about 80% of hepatic tissue volume (in both humans and rats). BECs of all bile duct segments in the liver constitute only about 3-5% of total liver nuclear population and less than 1% of total liver mass. Hepatocytes and BECs belong to the epithelial cell.

For a long time, it has been discussed whether there are liver stem cells in the postnatal liver. There are two arguments that do not support the existence of liver stem cells in the postnatal liver. First, the physiological liver cell renewal is very slow. It was concluded that only one of 20,000 to 40,000 liver cells is dividing at any given time and the liver replaced itself in approximately 1 to 2 years. Up to now, there have been no strong evidence that liver stem cells are involved in liver cell renewal. Second, liver cells are not end-staged differentiated cells. Although hepatocytes are usually in proliferatively quiescent state, they are capable of entering the cell growth cycle once there is loss of liver parenchyma. The typical example is that liver regeneration after a 70% liver resection in rats is followed by proliferation of mature liver cells. Thus it seems unnecessary for liver stem cells to exist in mature liver.

However, more and more studies have suggested that cells of stem cell potential exist in the liver, and these cells may be recruited to replace damaged liver cells when the injurious process to the liver is extensive or the injurious agent also inhibits proliferation of hepatocytes.

In 1937, Kinosita first reported discovery of small round cells in livers of rats exposed to the carcinogen „butter yellow", and in 1956 Farber coined the term „oval cells" for this kind of small cells. A popular model (AAF/PH) to generate oval cells is to feed animals with 2-acetylaminofluorence (AAF, which inhibits the proliferation of hepatocytes) before and after a two-thirds partial hepatectomy. Oval cells are classically characterized by ovoid nuclei and scanty basophilic cytoplasm. They arise in the periportal area and can be seen proliferating and migrating from the portal tract areas into liver parenchyma. Oval cells share similar phenotypes with BECs (CK 7, CK 19, CK 8, CK 18), however, they also contain markers normally seen in fetal liver cells, such as albumin, AFP, CK14, stem cell factor and its receptor (c-kit). OV-1 and OV-6 are two monoclonal antibodies that recognize oval cells. Oval cells have been shown to differentiate into hepatocytes or biliary cells both in vivo and in vitro (Golding et al. 1995) (Dabeva and Shafritz

1993; Germain et al. 1988; Lazaro et al. 1998). Thus, oval cells are suggested to derive from a putative stem cell population in rodent liver when the replicative capacity of the hepatocytes is greatly impaired.

In human diseased liver, oval-like cells were also discovered. Examination of the cell types involved in human ductular reactions has revealed small oval-like cells (small cells, ductular hepatocytes, small epithelial cells) in periportal zones. Ductular reaction is referred to the increase in ductular structures in the vicinity of portal tracts, accompanied by inflammatory cells and periductular fibrosis. Ductular reaction is a feature of many forms of human liver diseases, including regeneration, chronic cholestasis and cancer.

In 1992, De Vos described the presence of „small cells“ in the periportal area in human liver specimen with chronic ductular reaction (De Vos and Desmet 1992). These cells had an oval shape with an oval or oblong nucleus. Under electron microscope, they could be divided into three types. Type I cells represented the most immature progenitor cells, type II cells (intermediate bile duct-like cells) showed features of bile-duct cell differentiation, and type III cells (intermediate hepatocyte-like cells) displayed characteristics indicating hepatocellular differentiation. In 1996 (Roskams et al. 1996) and 1998 (Roskams et al. 1998), Roskams again described similar heterogeneous small cells in focal nodular hyperplasia (FNH) and regenerating human liver after submassive necrosis. These cells are immunoreactive for chromogranin-A (immature progenitor cells and intermediate hepatocyte-like cells), CK 7 and CK 19 (immature progenitor cells and intermediate bile duct-like cells) and OV-6 (all small cells).

Demetris (Demetris et al. 1996) and Haque (Haque et al. 1996) independently identified and characterized ductular hepatocytes (DHs) after submassive hepatic necrosis in human. They had a nuclear/cytoplasmic ratio that was higher than BECs or mature hepatocytes, and were located at the interface between the periportal connective tissue and liver cell plates. Immunohistochemical analysis of the highly proliferative DHs compartment shows it to be a heterogeneous population with unique phenotypic features. Although a subpopulation of DHs share phenotypic features with mature bile duct epithelium (CK19 positive) or hepatocytes (HepPar 1, albumin and alpha1 anti-trypsin positive), they are also clearly different from both populations. Moreover, occasional DHs also co-expressed HepPar1 and CK19, indicative of both hepatocyte and ductular differentiation.

Ruck described a population of small epithelial cells (SECs) that exhibit morphological and immunophenotypic features of the rodent oval cells in human hepatoblastoma (Ruck et al. 1996) and extrahepatic biliary atresia (Xiao et al. 1999). SECs co-express CK 7 (a marker of biliary differentiation) and albumin (a marker of hepatocytic differentiation), and also express oval cell associated antigens OV-1 and OV-6. SECs have also been demonstrated in various other liver

diseases, including alcoholic cirrhosis, chronic hepatitis and hepatocellular carcinoma, but not in the normal liver (Crosby et al. 1998a; Crosby et al. 1998b; Hsia et al. 1992).

In summary, oval-like cells in human liver were discovered mostly in conditions under severe hepatic injury, or chronic and persistent hepatic injury, or hepatic carcinogenesis. Thus, it can be speculated that, as in the rodent liver, activation of the putative stem cell pool in the human liver takes place only if the replicative capacity of the hepatocytes is greatly impaired by endogenous or exogenous noxious stimuli.

### 1.3. Putative location of liver stem cells in the liver

Most investigators have generally accepted the hypothesis that there are cells in the liver with stem cell potential (Crosby and Strain 2001). However, the exact location of liver stem cells in the liver is unclear. As oval cells share similar phenotypes with BECs and organize in ductular structure, the biliary tree has always been hypothesized to contain liver stem cells (Golding et al. 1996) (Alison and Sarraf 1998; Alison et al. 1996b; Sigal et al. 1992). It has been demonstrated that liver damage in the rat induces hepatic stem cells from biliary epithelial cells (Alison et al., 1996a). With the cholangiogram method, the continuity between duct-like structures formed by oval cells and pre-existing biliary tree had also been demonstrated (Dunsford et al. 1985) (Lenzi et al. 1992). Another convincing evidence is the inhibition of oval cell activation when bile ducts were damaged by 4,4‘-diaminodiphenylmethane (DAPM) (Petersen et al., 1997). The anatomic region that connects bile canaliculi (formed by hepatocyte) and bile ductule (the smallest branch of bile duct, containing biliary cells) is named canaliculo-ductular junction (CDJ), or canals (openings or orifices) of Hering. This junction is believed to be the location of the putative liver stem cell (Sell 1990) (Lemire and Fausto 1991). However, cells located within or in contact with the portal stroma (Yavorkovsky et al. 1995), small non-descriptive cells around cholangioles, the periductular cells (Sell 1993), and small interlobular ducts all have been proposed as candidates for the site of the stem cells (Nomoto et al. 1992).

### 1.4. The history of isolation and culture of hepatocytes and intrahepatic BECs

In 1967, Howard and Pesch (Howard et al. 1967) first used collagenase to disaggregate adult rat liver tissue, with the resultant of successful isolation of viable and functional hepatocytes. Seglen (Seglen 1976) refined this technique by perfusing the liver with a divalent cation free buffer before proteolytic digestion of the matrix with collagenase. This extra step effectively loosens the desmosome and hemi-desmosome junctions between the adjacent cells and extracellular matrix. Since then the so called two-step collagenase perfusion technique has become the „standard“ protocol for hepatocyte isolation.

From 1977, several laboratories have reported on the purification and culture of intrahepatic BECs from the rat liver after bile duct ligation (Sirica et al. 1990). Intrahepatic BECs represent only about 3-5% of total cells in the liver, and induction of compensatory proliferating bile ductules by bile duct ligation of rat liver can expand BECs population by up to 100 fold and generate sufficient cells for research (Sirica et al. 1990). Perfusion of rat liver in situ with digestive enzymes such as collagenase generates a heterogeneous mixture of cell types which requires further purification of BECs. Several techniques including differential density gradient centrifugation, centrifugal elutriation, fluorescence activated cell sorting, and immunological selection methods have been used either singly or in combination to produce a population enriched with BECs. The degree of enrichment for different procedures is variable ranging from less than 75% to greater than 95% (Joplin 1994).

In 1982, the isolation, in high yield and viability, of human hepatocytes from wedges of liver tissue was reported using the adapted two-step rat liver perfusion procedure (Strom et al. 1982). Adult human hepatocytes in primary culture can survive for some time in vitro, and have been used for metabolic studies and toxicity assays of a variety of chemicals. However, human hepatocytes in primary culture normally quickly lose their capacity for differentiated function and have limited capacity for replication. Lechner et al (1989) suggested that there was evidence for a „stem“ cell population in the adult human liver based on the finding that isolated hepatocytes could grow in serum free medium, at least for a limited time period. However, addition of hormones and growth factors provides greater growth potential.

In 1989, Joplin reported by using a monoclonal antibody (anti-HEA) which recognizes a specific antigen (human epithelial antigen, HEA) on the surface of BECs to positively purify human intrahepatic BECs from a semi-pure differential density centrifugation product (Joplin et al., 1989). Human intrahepatic BECs express the hepatocyte growth factor (HGF) receptor, c-Met, and respond to HGF. In the growth medium containing HGF, human BECs may sustain proliferation for 3 to 5 months and permit serial passages (Joplin et al. 1992). Intrahepatic BECs in culture express the typical phenotype, such as CK 7, CK 19 and γ-GT, and they also have the epithelial cell markers, such as CK 8 and CK 18. Intrahepatic BECs have a great potential to proliferate, but the origin of proliferating cells is not totally clear. Although the cells lining in the interior of large and medium sized bile ducts have been thought as their origin, it is also suspected that some of them possibly originate from the bile ductule, which is the smallest branch of the biliary system and believed to be the location of the putative liver progenitor or stem cell.

### 1.5. Aim of the study

As stated above, more and more investigators have believed that there are some cells with stem cell potential in the liver, and they may exist in biliary tree of rodent animal. Oval-like cells were also found in a variety of human liver diseases, and it has been suggested that human biliary tree may also contain liver stem cells. However, there has been no report to identify this. On the other hand, human intrahepatic BECs have a great potential to proliferate in vitro, but the origin of proliferating cells is not quite clear. It might contain some putative liver stem cells in human biliary tree.

In order to identify these hypotheses, human intrahepatic BECs were first isolated and cultured, and then characterized with different liver stem cells markers at both mRNA and protein level. Double-fluorescent immunostaining was used to check whether these cells co-express hepatocyte marker and biliary cell marker. The study includes: 1. Isolation and culture of human hepatocytes and intrahepatic BECs. 2. Morphological and phenotypic characterization of the cultured cells with phase contrast microscope, fluorescence activated cell sorting (FACS), reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry technique. 3. Measurement of lidocaine metabolism function of hepatocytes and BECs. 4. Measurement the effect of growth factors on the proliferation of BECs.

# 2. MATERIALS AND METHODS

## 2.1. Materials

### 2.1.1. Instruments

| | |
|---|---|
| Refrigerator 4 °C | Liebherr, Germany |
| Refrigerator -20 °C | Kirsch, Bosch, Germany |
| Refrigerator -80°C | NUAIR, Zapf instruments, Sarstedt |
| Cell culture incubator | Heraeus, Düsseldorf |
| Phase contrast microscope | Nikon, TMS, Japan |
| Fluorescence microscope | Nikon Elipse TS100, Japan |
| Confocal fluorescence microscope | Olympus BX 60, Japan |
| Table centrifuge | Heraeus, Düsseldorf |
| | Sigma 3K20, Osterode/Harz |
| Masterflex Digital Drive | Cole-pamer instrument Company, Illinois |
| Waterbath | Julabo, U3/7, Seelbach |
| Cell dissocitaion sieve | Sigma, USA |
| Magnetiser | Dynal A.S. Oslo, Norway |
| RNA/DNA Calculator | LKB Biochrom, England |
| Spektraphotometer PM6 | Zeiss, Oberkochen |
| GeneAmp PCR system 9600 | Pekin Elmer, USA |
| Electrophoresis equipment | Biometra PP3000, USA |
| Electrophoresis bath | Keutz, Reiskirchen |
| DNA-Gelcomb | DNA Subcell, Biorad, München |
| Protein-Gelcomb | Mini Protean II, Biorad, München |
| Proteintransfectcomb | Fastblot B33, Biometra, Göttingen |
| Geldocumentation | Biostep labor & systemtechnic GmbH, |
| Pipetting-tool | Jahnsdorf |
| Pipettierhilfen | Pipetus, Hirschmann Laboratory, Eberstadt |
| Microliterpipette | 1-10µl, 10-100µl, 100-1000µl, Eppendorf, |
| | Hamburg |
| | 0.1-2µl, Pipetman P2, Abimed, Langenfeld |
| Electronic Balance | MP-3000, Chyo, Japan |
| | Mettler AT261, Swiss |

| | |
|---|---|
| Magnetic stirrer | Combimag RET, IKA-Labortechnik, Staufen, i.Br. |
| Mini Shaker | MS1, IKA-Labortechnik |
| Circular Shaker | KS125, IKA-Labortechnik |
| Horizontal shaker | Vibrax-VXR, IKA-Labortechnik, Staufen i. Br. |
| FACS measure instrument | FACScan, Becton-Dickinson, Heidelberg |
| Elisa-measure instrument | LambdaE, MWG-Biotech, Ebersberg |
| pH-Meter | pH-Meter 765, Calimatic, Germany |
| Cell smear machine | Auto smear CF-12DE, SAKURA |
| TDx Analyzer | Abbott Laboratories, Illinois |

**2.1.2. Consumer Material**

| | |
|---|---|
| Measure cylinder 100, 250, 500, 1000ml | Schott Glaswerke, Mainz |
| Beaker glass 250, 400, 600ml | Schott Glaswerke, Mainz |
| Erlenmeyer flask | Schott Glaswerke, Mainz |
| Sterile pipet 10µl | Greiner, Frickenhausen |
| Sterile pipet 100µl, 1000µl | Biozym Diagnostik GmbH, Hess. Oldendorf |
| Pipet tips 10µl, 100µl, 1000µl | Eppendorf, Hamburg |
| Pipet tips 2µl | Abimed, Langenfeld |
| Gel loading tip | Biozym Diagnostik GmbH, Hess. Oldendorf |
| Falcon Pipet 2, 5,10, 25, 50ml | Becton Dickinson Labware, NJ, USA |
| Centrifuge tube 15, 50ml | Greiner, Frickenhausen |
| Vials 0,5 / 1,5 / 2ml | Eppendorf, Hamburg |
| 96-well Elisa-plate | Costar Europe, Badhoevedrop / NL |
| Tissue culture Plates (24 well, 12 well, 6 well) | Greiner, Frickenhausen |
| Tissue culture Flasks (25$cm^2$, 75 $cm^2$, 182 $cm^2$) | Greiner, Frickenhausen |
| Petri Dishes (60/15mm , 94/16mm, 145/20 mm) | Greiner, Frickenhausen |
| Nitrocellulose | Schleicher & Schuell, Dassel |
| Cell scraper | Greiner, Frickenhausen |
| Millipore Bacterial Filtrator | Molsheim, France |

| | |
|---|---|
| Nalgene Filtration Products 100, 500, 1000ml | Nalge Nunc international, New York |
| Sterile Specimen Container | Becton Dickinson Labware, NJ, USA |

### 2.1.3. Chemical

#### 2.1.3.1. Cell isolation and culture

| | |
|---|---|
| Hanks' Balanced Salt Solution (HBSS) without Calcium & Magnesium | Biochrom KG, Berlin |
| Dulbecco's Modified Eagle Medium (DMEM) | PAA, Austria |
| DMEM / HAM's F-12 | PAA, Austria |
| PBS with Calcium & Magnesium | Biochrom KG, Berlin |
| PBS without Calcium & Magnesium | Biochrom KG, Berlin |
| William's Medium | Biochrom KG, Berlin |
| 1M HEPES Buffer | Biochrom KG, Berlin |
| 200mM L-Glutamine | Biochrom KG, Berlin |
| 10mg/ml Gentamicin | Biochrom KG, Berlin |
| 4mg/ml Collagen G (From Bovine Calf Skin) | Biochrom KG, Berlin |
| Percoll (1. 124g/ml) | Biochrom KG, Berlin |
| EGTA | Sigma, Deisenhofen |
| $MgCL_2$ | Sigma, Deisenhofen |
| Tissue Adhesive | Braun, Germany |
| Insulin | Sigma, Deisenhofen |
| Cholera toxin | Calbiochem |
| Hydrocortisone | Sigma, Deisenhofen |
| Triiodothyronine | Sigma, Deisenhofen |
| HGF | R & D, UK |
| EGF | Sigma, Deisenhofen |
| 10000 units/ml penicillin and 10000 mg/ml streptomycin | Life Technologies, Karlsruhe |
| Fetal Bovine Serum (FBS) | Gibco BRL, Scotland |
| Collagenase IA (467 units/mg) | Sigma, Deisenhofen |
| Collagenase IV (449 units/mg) | Sigma, Deisenhofen |
| Collagenase XI (1240 units/mg) | Sigma, Deisenhofen |

| | |
|---|---|
| TGF-$\beta_1$ | Sigma, Missouri |
| 0.5g/l Trypsin and 0.2g/l EDTA in HBSS | Sigma, UK |
| EDTA | Merck, Darmstadt |

**2.1.3.2. RT-PCR**

| | |
|---|---|
| Trizol Reagent | Life Technologies, New York |
| Isopropanol | Merck, Darmstadt |
| 2-Mercaptoethanol | Merck, Darmstadt |
| Chloroform | Merck, Darmstadt |
| Ethanol 100% | Serva,Heidelberg |
| DEPC-treated Water | Ambion |
| Total RNA Isolation Reagent | Life Technologies, New York |
| RNA Reversetranscription Kit | Promega, USA |
| Rneasy mini Kit | Qiagen, Germany |
| Taq Polymerase and 10X PCR Buffer | Roche, Manheim |
| 10mM dNTP | Boehringer, Manheim |
| Primers | Carl Roth, Karlsruhe |
| Loading buffer | Fermentas, Lithuania |
| DNA ladder | Fermentas, Lithuania |
| Agarose | Pharmacia, Denmark |
| Ethidiumbromide (1mg/ml) | Sigma-Aldrich, Deisenhofen |

**2.1.3.3. FACS and immunohistochemistry**

| | |
|---|---|
| Fix & Perm cell permeabilization kit | Dianova, Hamburg |
| Formaldehyde 37% | Sigma-Aldrich, Deisenhofen |
| Methanol | Roth, Karlsruhe |
| Goat serum | Sigma-Aldrich, Deisenhofen |
| Pig serum | Nordic, Germany |
| Peroxidase Blocking Reagent | DAKO, Denmark |
| Aminomethylpropandiol-buffer | Merck, Darmstadt |
| Tris-buffer PH 9.7 | Merck, Darmstadt |
| NaCl | Merck, Darmstadt |
| Levamisole | Sigma, Steinheim |
| Naphthol-AS-Bi-Phosphat | Sigma, Steinheim |
| DMF (Dimethylformamid) Solution | Merck, Darmstadt |

| | |
|---|---|
| Neufuchsinlsg 5% | Chroma |
| Natriumnitritlsg 4% | Sigma, Steinheim |
| Ethylcarbazole | Sigma, Steinheim |
| Aceton | Merck, Darmstadt |
| Dimethylsulfoxid solution | Merck, Darmstadt |
| 3% $H_2O_2$ | Merck, Darmstadt |
| Mayer's Hematoxylin | Merck, Darmstadt |
| Glyceringelatine | Merck, Darmstadt |

**2.1.3.4. Antibodies**

| | |
|---|---|
| HEA (monoclonal, $IgG_1$) | Progen, Heidelberg |
| Cytokeratin 7 (monoclonal, $IgG_1$) | Progen, Heidelberg |
| Cytokeratin 8 (monoclonal, $IgG_1$) | Progen, Heidelberg |
| Cytokeratin 18 (monoclonal, $IgG_1$) | Progen, Heidelberg |
| Cytokeratin 19 (monoclonal, $IgG_{2b}$) | Progen, Heidelberg |
| Cytokeratin 14 (monoclonal, $IgG_1$) | Progen, Heidelberg |
| Vimentin (monoclonal, $IgG_{2a}$) | Progen, Heidelberg |
| Desmin (monoclonal, $IgG_1$) | Progen, Heidelberg |
| Rabbit anti-human Von Willebrand Factor (polyclonal) | DAKO, Denmark |
| Pan-CK (monoclonal) | Linaris, Germany |
| Fix and Perm Cell Permeablization Kit | Dianova, Hamburg |
| ASGPR (monoclonal, $IgG_{1,k}$) | Calbiochem, Darmstadt, Germany |
| ASGPR (polyclonal) | Eurogentec, Seraing, Belgium |
| Alpha1-antitrypsin (monoclonal, $IgG_2$) | Chemicon, Temecula, CA |
| Alpha1-antitrypsin (polyclonal) | DAKO, Denmark |
| Albumin (monoclonal, $IgG_{2a}$) | Sigma, Missouri |
| AFP (monoclonal, $IgG_{2a}$) | Sigma, Missouri |
| AFP (polyclonal) | Nordic, Germany |
| c-kit (monoclonal) | DAKO, Hamburg |
| Chromogranin-A (monoclonal) | Linaris, Germany |
| Chromogranin-A (monoclonal, $IgG_{1k}$) | Progen, Heidelberg |
| FITC-conjugated Goat anti-mouse IgG | Dianova, Hamburg |
| FITC-conjugated Goat anti-rabbit IgG | Dianova, Hamburg |
| Goat Anti-Mouse $IgG_1$ (FITC conjugate) | Southern Biotechnology Associates, Inc. |

| | |
|---|---|
| | Alabama |
| Goat Anti-Mouse $IgG_{2a}$ (TXRD conjugate) | Southern Biotechnology Associates, Inc. Alabama |
| Sheep anti-mouse $IgG_1$ (Fc) coated Dyna beads | Dynal A.S. Oslo, Norway |
| Mono-AP-Goat anti Mouse IgG | DAKO, Hamburg |
| Goat anti Rabbit Immunoglobin | Nordic, Germany |
| PAP-complex | Nordic, Germany |

**2.1.3.5. Others**

| | |
|---|---|
| MTT | Sigma, Deisenhofen |
| Lidocaine | Astra GmbH, Wedel |
| TDx/TDxFLx Lidocaine Assay kit | Abbott Laboratories, Illinosis |

## 2.2. Patients

As depicted in the next table, diseased liver tissue was obtained from explanted livers after liver transplantation (No. 1, 3, 7, 8,9), and normal tissue were obtained from liver resections (No 2, 6 and 10 to 20) and donor liver (No. 4, 5) when it exceeded the surgical requirement. All of the specimens were kindly provided by the Department of General Surgery and Transplantation at this hospital (Director: Prof. Dr. med. Dr. h.c. mult. Christoph E. Broelsch).

**Table 2.1 General information of the patients and derived cells**

| No. | Age (years) | Sex | Diagnosis | Derived cells |
|---|---|---|---|---|
| 1 | 1 | female | Oxaluria | BECs |
| 2 | 1.5 | male | hepatoblastoma | BECs, hepatocytes |
| 3 | 1 | female | Carbamyl-phosphat-synthetase-defect | BECs |
| 4 | 10 | female | donor | BECs |
| 5 | 6 | female | donor | BECs |
| 6 | 9 | female | liver neuroendocrine carcinoma | BECs |
| 7 | 44 | female | Autoimmune hepatitis (AIH) | BECs |
| 8 | 64 | female | primary biliary cirrhosis (PBC) | BECs |
| 9 | 55 | female | primary biliary cirrhosis (PBC) | BECs |
| 10 | 60 | male | cholangiocarcinoma | BECs |
| 11 | 71 | female | hepatocellular carcinoma | hepatocytes |
| 12 | 33 | female | focal nodular hypertrophy | hepatocytes |
| 13 | 62 | female | cholangiocarcinoma | hepatocytes |
| 14 | 45 | female | focal nodular hypertrophy | hepatocytes |
| 15 | 71 | male | metastatic liver carcinoma from colon | hepatocytes |
| 16 | 72 | male | metastatic liver carcinoma from rectum | hepatocytes |
| 17 | 51 | male | hepatocellular carcinoma | hepatocytes |
| 18 | 52 | female | metastatic liver carcinoma from colon | hepatocytes |
| 19 | 68 | male | metastatic liver carcinoma from colon | hepatocytes |
| 20 | 60 | male | hepatocellular carcinoma | hepatocytes |

## 2.3. Methods

### 2.3.1. Isolation and culture of human hepatocytes

Shortly after liver resection, 10-20 g of the normal tissue near the edge of liver was cut in wedged shape. The tissue was kept in a sterile beaker containing DMEM and brought from the operation room to the cell culture room. Before hepatocyte isolation, the tissue was stored in DMEM at 4 °C for less than 5 hours. All procedures were done under aseptic conditions.

**Perfusion of the liver tissue**

1) The liver tissue was put into one Petri dish (145 mm in diameter). One of the exposed blood vessels on the cut surface was carefully selected for efficient perfusion, and cannulated with the catheter operated by the Masterflex Digital Drive. The remaining exposed vessels were closed by the tissue adhesive.
2) Firstly, the liver tissue was perfused with 300-400 ml 4°C HBSS (containing 0.002% Gentamycin and 0.2M HEPES) to expel the blood inside the liver pieces. The perfused HBSS solution in the dish was removed completely before the next step.
3) Secondly, the liver tissue was perfused with 120ml HBSS containing 0.5mM EGTA. The first part of perfused HBSS (about 20 to 40 ml) was discarded, then the tissue was continually perfused for 15 minutes. The circulating tube (catheter) was warmed to keep the temperature of perfusion solution around 35°C.
4) Thirdly, the liver tissue was perfused with 120 ml HBSS containing 5 mM $CaCL_2$ and 0.0625% Collagenase IV. The first part of the perfused HBSS (about 20 to 40 ml) was also removed, and then the tissue was continually perfused for 10 to 15 minutes (depending on the texture of liver tissue). The perfused enzyme solution was also maintained about 35°C. The enzyme solution in the dish was removed completely before next step.
5) In the end, the capsule of the tissue was cut, and the digested tissue was washed with 4°C HBSS and passed through the cell dissociation sieve (mesh 60) to remove the undigested connective tissue. The cell suspension was made up to 300ml with HBSS and transferred to six 50ml centrifuge tubes.

**Wash of the hepatocytes**

1) The cell suspension was spinned at 300 rpm (30g) for 10 minutes at room temperature.
2) The supernatant was carefully decanted to avoid the loss of the hepatocytes at the bottom.
3) 50ml HBSS was added to each tube, and the tube was shaken slightly to wash the cells.
4) The cell suspension was again spinned at 400rpm (35g) for 8 minutes at room temperature.
5) Steps 3 to 5 were repeated until the supernatant was clear.

**Plating of hepatocytes**

1) The hepatocyte pellet was pooled into 2 or 3 tubes, and re-suspended with plating medium (DMEM/HAM's F-12 with 10% FCS, 40units/ml penicillin, 40µg/ml streptomycin).
2) The yield and viability of the hepatocytes was determined with the hemacytometer. The cell density was adjusted to 500.000/ml before plating.
3) The hepatocytes were plated in tissue culture plates that had been coated with 0.5mg/ml collagen G. (The well of plate was coated with collagen G for 30 minutes at room temperature and then washed with PBS.) For the 24-well plate, the 12-well plate and the 6-

well plate, each well was plated with 250.000, 500.000 and 1.500.000 of hepatocytes, respectively.

4) The cells were kept in an incubator at 37 °C with 5% $CO_2$.
5) After 20 to 30 minutes, viable hepatocytes adhered, and the supernatant was removed to eliminate the floating dead cells. The medium for the culture was DMEM/HAM's F-12 with 2-5% FCS, 40unit/ml penicillin and 40µg/ml streptomycin.
**6)** The medium was changed every 24 hours and the growth of hepatocytes was observed under phase contrast microscope.

### 2.3.2. Isolation and culture of human intrahepatic BECs

Normal liver tissue was obtained from liver resection or from liver donors (as stated before). Diseased liver tissue was obtained from explanted liver during liver transplantation. The tissue was stored in DMEM or UW solution at 4 °C within 36 hour before isolation of BECs. All procedures were done under aseptic conditions.

1) 30-50 g of liver tissue were cut into 2-3 $mm^3$ pieces, and the liver pieces were put into a beaker with 45ml PBS (with $Ca^{2+}$ and $Mg^{2+}$).
2) 5 ml of Collagenase IA (10mg/ml) was added into the beaker to give a final concentration of 1 mg/ml collagenase IA.
3) The beaker was placed at 37 °C incubator for 30 to 60 minutes and kept shaking with a circular shaker. The duration depended on the texture of the liver tissue.
4) The enzyme digesting suspension in the beaker was passed through the cell dissociation sieve (mesh 40) and kept the undigested tissue.
5) The undigested liver tissue was cut into even finer pieces.
6) The filtrated solution was spinned at 2000rpm (550g) for 10 minutes at room temperature. The supernatant with collagenase was used to digest the liver tissue again at 37 °C for 15 to 30 minutes, and the cell pellet was kept in PBS (The PBS used in this and the next steps was without $Ca^{2+}$ and $Mg^{2}$).
7) The solution in the beaker was passed through the sieve again and the digested tissue was washed in the sieve with PBS. Then the filtrated solution was combined with the solution in step 6) and made up to 200 ml with PBS.
8) The cell suspension was dispensed into four 50-ml tubes, and the tubes were spinned at 2000 rpm for 10 minutes at room temperature.
9) The cell pellet was washed with PBS until the supernatant was fairly clear.
10) The pellet was collected into one tube and re-suspended with 24 ml PBS.

11) Eight 15-ml centrifuge tubes with 3ml of 68% Percoll solution (1.04g/ml) on the bottom and 3ml of 29% Percoll solution (1.09g/ml) on the top were prepared.
12) Gently, 3 ml of cell suspension was pipetted onto the top of each Percoll gradient.
13) The cells were spinned at 2500rpm (800g) for 30 minutes at room temperature. The brake of the centrifuge was turned off during the centrifugation.
14) The top layer of „debris“ was discarded.
15) The differential band of cells were pipetted into one 50-ml tube and made up to 50ml with PBS.
16) The cells were spinned at 2000rpm for 10 minutes.
17) The cell pellets were suspended with PBS and transferred to one 15 ml-tube.
18) The cell pellets were washed with PBS repeatedly until the supernatant was fairly clear.
19) The cell pellets were re-suspended in 0.45ml PBS in a 15-ml tube and added into 50µl of aliquot HEA (2.5µg).
20) The tube was placed at 37 °C for 30 minutes and shaken every 10 minutes to mix the solution.
21) The tube was made up to 10ml with PBS and spinned at 2000rpm for 10 minutes.
22) The pellet was re-suspended in 0.5ml cold PBS and 10µl of sheep anti-mouse IgG1 (Fc) coated Dyna beads ($4\times10^7$) were added into it.
23) The tube was placed at 4 °C refrigerator for 30minutes and shaken occasionally (2 to 5 times).
24) The tube was made up to approximately 5ml with PBS and placed in the magnetiser.
25) After 2 minutes, the supernatant was removed to one 15-ml tube.
26) The last two steps were repeated two more times and the supernatant was collected into the same tube.
27) The cells binding with the beads were suspended in 5ml plating medium (table 2.2) and plated in one 25-$cm^2$ flask coated with collagen G.
28) The above supernatant was spinned at 2000rpm for 10 minutes, and the cells (not binding with the beads) were re-suspended with 5 ml plating medium. Also, these cells were plated in one 25-$cm^2$ flask coated with collagen G.
29) The cells were kept in an incubator at 37 °C with 5% $CO_2$.
30) When the cells had adhered after about 24 to 72 hours, the medium was changed into growth medium (table 2.2). Later the growth medium was changed every 3 days or longer, depending on the growth speed of the cells.

**Table 2.2 Prescription of plating medium and growth medium**

| Contents | Plating medium | Growth medium |
|---|---|---|
| DMEM | 45% | 47.5% |
| Ham's F12 | 45% | 47.5% |
| FCS | 10% | 5% |
| Insulin | 5 μg/ml | 5 μg/ml |
| HGF | 0 | 10 ng/ml |
| EGF | 10 ng/ml | 10 ng/ml |
| Cholera toxin | 10 ng/ml | 10 ng/ml |
| Hydrocortisone | 2 μg/ml | 2 μg/ml |
| Triiodothyronine | 2 nM | 2 nM |
| Streptomycin | 40 μg/ml | 40 μg/ml |
| Penicillin | 40 units/ml | 40 units/ml |

**Passage of BECs**

When BECs were confluent in the flask, they were passaged with the next method.

1) The medium in the flask was removed completely, and then the cells were washed with PBS to get rid of FCS.
2) 2ml of 0.1g/l trypsin and 0.04g/l EDTA (diluted in 0.1% PBE) was added into one 25-$cm^2$ flask, and the flask was placed in the incubator (37°C) for about 5 minutes. The process of enzymatic digestion was checked under the microscope. When the cells became round and detached, the digestion was stopped by adding 2 ml DMEM with 5% FCS.
3) The cell suspension was collected and spinned at 1400 rpm for 4 minutes.
4) The cell pellets were re-suspended with plating medium and the number of BECs was counted.
5) The cells were plated in the culture plate or flask at some density according to the aim of experiment.
6) After the cells adhered, the cells were cultured with growth medium.

**Freezing and thawing of the BECs**

During passage of BECs, the cells were re-suspended in 1 million per ml freezing medium (containing 50% DMEM with F12, 40% FCS and 10% DMSO). The cells were first kept at –80 °C refrigerator and then transferred to liquid nitrogen. For thawing of the cells, the cells in the freezing tube was taken out from the liquid nitrogen and thawed at room temperature. Then plating medium was used to plate the cells.

### 2.3.3. Fluorescence activated cell sorting (FACS)

One day after plating hepatocytes, the expression of CK 8, CK 18, vimentin, ASGPR by hepatocytes were checked with FACS method. BECs were also checked for the expression of CK 7, CK 8, CK 18, CK 19, vimentin, desmin with this method.

**Detachment of the cells (Take the cells plated in the 12-well plate as example)**

1) The medium was removed and the cells were washed with PBS.
2) 0.5ml of collagenase XI (0.02%) was added into each well and incubated with the cells at 37°C for 15 minutes. Then 0.5ml of 0.1% PBE was added into each well and incubated for another 5 minutes at 37°C.
3) The solution was pulled up and down with plastic pipette for several times to help detach the cells.
4) The cell suspension was collected into one tube and spinned at 1400 rpm for 4 minutes.
5) The cells were washed with PBS and divided into several equal parts for staining.

**Staining of the cells** (with Fix & Perm cell permeabilization kit)

1) 100µl of solution A (for fixation) in the kit were taken to fix the cells in the tube at room temperature for 15 minutes.
2) The cells were washed with 2ml PBS (the tube was spinned at 1400 rpm for 4 minutes, and the below wash step was the same as this)
3) 100µl of the first antibody were added into the tube and the tube was vortexed to mix the cells with the first antibody. ASGPR (1:100, polyclonal) was diluted in PBS, and CK 7 (1:3), CK 8 (1:3), CK 18 (1:3), vimentin (1:3) were diluted in solution B (for permeablization), The cells were incubated with the primary antibody for 30 minutes at 4°C.
4) The cells were washed to remove the remaining first antibody.
5) 100µl of the second antibody (FITC conjugated goat anti-mouse IgG, 1:40; FITC conjugated goat anti-rabbit IgG, 1:100) were added into the tube and the tube was vortexed to mix the cells with the second antibody. The incubation time was also 30 minutes at 4°C.
6) The cells were washed to remove the remaining second antibody.
7) The cells were re-suspended with 0.5ml of 1% formaldehyde in PBS.
8) The cells were stored at 4°C in the dark before measurement.

**Measurement**

FACScan, Becton-Dickinson was used to measure the result.

### 2.3.4. Indirect immunofluorescence staining

Hepatocytes, BECs grown in the 24-well plate had been checked with this method.

**Fixation of the cells**

1) The medium in the well was aspirated completely.
2) The cells were washed 1 time with 4°C PBS for 5 minutes.
3) 0.25ml methanol that had been pre-cooled to -20 °C was added into each well.
4) The plate was put in -20 °C refrigerator for 6 minutes.
5) The methanol was aspirated completely.
6) The cells were washed 3 times with 4°C PBS for 15 minutes.

**Staining of the cells**

The primary antibodies, their working concentration and incubation time, and the stained cells are listed in next table.

**Table 2.3 The primary antibody used for indirect immunofluorescence**

| Antibody | Dilution | Incubation time | Stained cells |
|---|---|---|---|
| HEA ( $IgG_1$) | 1:10 | 1 hour at RT | BECs |
| CK 7 ( $IgG_1$) | 1:3 | 1 hour at RT | BECs |
| CK 8 ( $IgG_1$) | 1:3 | 1 hour at RT | Hepatocytes, BECs |
| CK 18 ( $IgG_1$) | 1:3 | 1 hour at 37°C | Hepatocytes, BECs |
| CK 19 ($IgG_{2b}$) | 1:3 | 1 hour at RT | BECs |
| CK 14 ( $IgG_1$) | 1:5 | 1 hour at RT | BECs |
| Vimentin ($IgG_{2a}$) | 1:3 | 1 hour at RT | BECs |
| Desmin ($IgG_1$) | 1:10 | 1 hour at RT | BECs |
| ASGPR ($IgG_{1,k}$) | 1:50 | 1 hour at RT | Hepatocytes, BECs |
| α1-antitrypsin ($IgG_2$) | 1:10 | 1 hour at RT | Hepatocytes, BECs |
| Albumin ($IgG_{2a}$) | 1:25 | 1 hour at RT | Hepatocytes, BECs |
| Factor VIII* | 1:200 | 1 hour at RT | BECs |

***** Polyclonal antibody, the others are all monoclonal antibodies.

1) PBS in the well was removed completely, and 100µl of 10% goat serum were added into each well. The serum was incubated with cells at room temperature (RT) for 15 minutes.
2) The cells were washed with PBS 3 times for 15 minutes.
3) 100µl of primary antibody were added into different wells. 100µl of PBS were used to replace the primary antibody as the negative control. The incubation time was listed in the above table.
4) The cells were washed 3 times with PBS for 15 minutes.
5) 100µl of the secondary antibody were added into each well. For primary monoclonal antibody, FITC conjugated goat anti-mouse IgG (1:50) was used as the second antibody;

for primary polyclonal antibody, FITC conjugated goat anti-rabbit (1:100) IgG was used as the second antibody. For the staining of albumin and vimentin, goat anti-mouse $IgG_{2a}$ (TXRD conjugated) was also used as the second antibody.

6) The cells were washed 3 times with PBS for 15 minutes.
7) The cells were covered with PBS and stored in 4°C refrigerator. The staining result was observed under fluorescence microscope.

**Double-fluorescent immunostaining**

Goat anti-mouse $IgG_1$ (FITC conjugate) and goat anti-mouse $IgG_{2a}$ (TXRD conjugate) were used in this experiment. Monoclonal antibodies against CK 7, CK 8 and CK 18 are all $IgG_1$ subclass, and monoclonal antibodies against albumin and vimentin are both $IgG_{2a}$ subclass. The co-expression of CK 7 and albumin, CK 7 and vimentin, CK 8 and albumin, CK 18 and vimentin by BECs were checked by this method.

1) The fixation of the cells and decrease of non-specific stain with 10% goat serum was the same as above.
2) Primary antibodies, CK 7 (1:3) and albumin (1:40), CK 8 (1:3) and albumin (1:40), CK 18 (1:3) and vimentin (1:3), CK 7 (1:3) and vimentin (1:3) were diluted in PBS. 100 µl primary antibodies were added into each well of 24-well plate, and 100µl of PBS were used to replace the primary antibodies as the negative control. The reaction was incubated at room temperature for 30 minutes.
3) The cells were washed 3 times with PBS for 15 minutes.
4) Second antibodies, goat anti-mouse $IgG_1$ (FITC conjugate, 1:100) and goat anti-mouse $IgG_{2a}$ (TXRD conjugate, 1:100) were diluted in PBS, and 100µl second antibodies were added into each of 24-well plate. The reaction was done at room temperature for 30 minutes.
5) The cells were washed 3 times with PBS for 15 minutes.
6) The result was detected under fluoresecent microscope (Olympus BS 51, Japan).

### 2.3.5. Reverse transcription polymerase chain reaction (RT-PCR)

The expression of albumin mRNA and AFP mRNA by human intrahepatic BECs were checked with RT-PCR method. Primary human hepatocytes, HepG2 cell line, Raji cell line were used as controls.

#### 2.3.5.1. RNA extraction from the monolayer cells

During the passage of BECs, HepG2 and Raji cells, they were plated in the Petri dish with 60mm in diameter for RNA extraction. Freshly isolated hepatocytes were directly plated in the same type of Petri dish.

**Homogenization**

1) The medium was removed completely and 1ml Trizol was added into each dish.
2) The cells were scraped into the Trizol with a sterile cell scraper.
3) The cells in Trizol were transferred to an autoclaved 1.5ml vial (microfuge tube).

**Phase Separation**

1) The cells were incubated in Trizol at room temperature for 5 minutes.
2) 0.2ml chloroform was added into the vial and it was shaken vigorously by hand for 15 seconds.
3) The reaction was incubated at room temperature for 2-3 minutes.
4) The vial was centrifuged at 12 000 g for 15 minutes at 4°C.
5) The upper (colourless) phase was pipetted off and put in new Eppis on ice.

**RNA Precipitation**

1) 0.5ml isopropanol was added into the Eppis and the Eppis was vortexed shortly.
2) The reaction was incubated on ice for 10 minutes.
3) The vial was centrifuged at 12 000 g for 10 minutes at 4°C.
4) The supernatant was removed immediately by pouring it out slowly in one motion and the last drop was blotted with paper tissue.

**RNA Wash**

1) The vial was put on ice and 1ml ice cold 75% ethanol was added into it. (The 75% ethanol was made with 100% ethanol and DEPC-treated $H_2O$.) The vial was vortexed shortly.
2) The vial was centrifuged at 7500 g for 5 minutes at 4°C.
3) The supernatant was removed as before and the vial was put on ice.
4) The vial was centrifuged again at 7500 g for 1 minute at 4 °C and then put on ice.
5) The rest of the supernatant was removed with an autoclaved 10ul gel-loading tip.
6) The RNA was dissolved in 50µl of DEPC-treated $H_2O$ and the RNA solution was freezed at -80°C. After one freeze-thaw cycle, the RNA would be in solution.

**RNA cleanup** (with Rneasy Mini Kit)

1) The sample was adjusted to a volume of 100 µl with RNase-free water, and 350 µl of Buffer RLT (with 10% ß-Mercaptoethanol) was added into it. The solution was mixed thoroughly.
2) 250µl of ethanol (100%) were added into the lysate and they were mixed well by pipetting.

3) The sample (700 µl) was applied to a Rneasy mini spin column sitting in a collection tube, and the tube was centrifuged for 15 seconds at 8000 g.
4) The Rneasy column was transferred into a new 2-ml collection tube, and 500µl of buffer RPE were added onto the column. The tube was centrifuged for 15 seconds at 8000 g to wash the RNA.
**5)** 500µl of buffer RPE were pipetted onto the Rneasy column, and the tube was centrifuged for 2 minutes at 14.000 rpm to dry the Rneasy membrane.
**6)** The Rneasy column was transferred into a new 1.5ml collection tube, and 30µl of RNase-free water were pipetted directly onto the Rneasy membrane. The tube was centrifuged for 1 minute at 10.000 rpm to elute the RNA.

**RNA calculation**

The purity, ratio of OD260/OD280 and concentration of isolated total RNA were measured with RNA/DNA Calculator (LKB Biochrom, England).

### 2.3.5.2. Reverse transcription

**Table 2.4 The composition of reverse transcription**

| | **Negative Control** | **reverse transcription** |
|---|---|---|
| 25mM $MgCL_2$ | 4µl | 4µl |
| RT 10 X Buffer | 2µl | 2µl |
| 10mM dNTP | 2µl | 2µl |
| RNAsin | 0.5µl | 0.5µl |
| 0.5µg/µl Oligo $(dT)_{15}$ | 1µl | 1µl |
| 20units/µl AMV-RTase | 0 | 0.75µl |
| DEPC treated water | make up to 20µl | make up to 20µl |

1) 2µg of total RNA were denatured in the Eppis at 70°C for 10 minutes
2) The vial with RNA was directly put on ice for no less than 5 minutes.
3) The vial was centrifuged at 4°C for 1 minute to let the evaporated water under the lid go to the bottom.
4) A reaction to synthesize the first strand of cDNA from the mRNA in total RNA was set up as below table.
5) The reaction was incubated at 42°C for 60 minutes.
6) The AMV-RTase was inactivated at 95°C for 5 minutes.
7) The Eppis was cooled to room temperature and centrifuged to let the evaporated water under the lid go to the bottom. The cDNA was stored in the vial at -20°C.

### 2.3.5.3. PCR

The sequence of the primers for GAPDH, albumin and AFP were listed in next table.

**The 2.5 The sequence of the primers used in this experiment**

| Primers | Sequence | Position in the cDNA |
|---|---|---|
| GAPDH-F* | 5'-CAA CTA CAT GGT TTA CAT GTT-3' | 120 - 140 |
| GAPDH-R# | 5'-ACT TGT GGT CAT GAG TCC TTC-3' | 514 - 534 |
| Albumin-F | 5'-GTG TTT CGT CGA GAT GCA CAC-3' | 60 - 80 |
| Albumin-R | 5'-AAG TTC ATC GAG CTT TGG CAA-3 | 606 - 626 |
| AFP-F | 5'-ACA CTG CAT AGA AAT GAA TAT-3' | 57 - 77 |
| AFP-R | 5'-TTC AGC TTT GCA GCA AGA TGG-3' | 567 - 587 |

* Forward Primer # Reverse Primer

**PCR**

A PCR reaction to amplify the targeted mRNA using cDNA as template was set up as below.

**Table 2.6 The compositions of PCR**

| | Negative Control | RT-PCR |
|---|---|---|
| Template | 2µl cDNA | 2µl cDNA |
| 10 X PCR Buffer | 5µl | 5µl |
| Forward primer (2µM) | 10µl | 10µl |
| Reverse primer (2µM) | 10µl | 10µl |
| Taq polymerase (5units/µl) | 0.25 µl | 0.25 µl |
| DNase free water | make up to 50µl | make up to 50µl |

**PCR Conditions**

**Table 2.7 The procedures for second stage of PCR**

| Products | Denaturation | Annealing | Extension | Number of cycles |
|---|---|---|---|---|
| GAPDH | 94°C/60 seconds | 60°C/60 seconds | 72°C/60 seconds | 25 |
| Albumin | 94°C/60 seconds | 60°C/60 seconds | 72°C/60 seconds | 35 |
| AFP | 94°C/60 seconds | 60°C/60 seconds | 72°C/60 seconds | 35 |

PCR was operated with GeneAmp PCR system 9600 (Pekin Elmer), and the process of PCR included 3 stages:

1) The first stage of PCR: Denaturation at 94°C for 5 minutes.
2) The second stage of PCR was as below table.
3) The third stage of PCR: Extension at 72°C for 10 minutes.

#### 2.3.5.4. Electrophoresis

1) 10µl PCR solution were taken and mixed with 2µl six-times loading buffer
2) The PCR products were run in 1% agarose gel (in TBE) containing 1mg/ml ethidium bromide.
3) The results were observed under the gel documentation equipment.

### 2.3.6. Immunohistochemistry

Several lines of cultured BECs in the flasks were detected the expression of Pan-CK, AFP, α1-antitrypsin, c-kit, chromogranin-A with immunohistochemistry technique (This part was done under the kind guide of Dr. med. Olaf Dirsch).

**Cell smear preparation** (with the help of Frau Birgit Linker)

The cells were detached with 0.2% PBE (incubation at 37°C for 10 minutes) or with the cell scraper, and single cells were collected onto a glass slide using a Cytospin cytocentrifuge. The cells on the slides were fixed with 95% ethanol. Heat-induced epitope retrieval was performed by boiling the slides with specimen in 97.7°C water for 40 minutes and cooling at room temperature for 20 minutes. Antibodies used in these experiments were listed in next table.

**Table 2.8 The primary antibodies used for immunohistochemistry**

| Antibody | Dilution | Duration |
|---|---|---|
| Pan-CK* | ready to use | 2hr. at 37°C |
| AFP* | 1:100 | 30min. at RT |
| α1-antitrypsin* | 1:50 | 30min. at RT |
| c-kit# | 1:50 | 30min. at RT |
| chromogranin-A# | ready to use | 2hr. at RT |

*polyclonal antibody # monoclonal antibody

#### 2.3.6.1. Powervision detection system

Pan-CK, c-kit and chromogranin-A were stained with Powervision detection system (with the help of Frau Ute Möser, Frau Michaela Kasimir and Frau Renate Kern).

**Procedure of stain**

1) The slides were taken out of the ethanol solution, and washed with "DAKO wash buffer" (The below wash buffer was the same as this).
2) The sample was incubated with "Background Reducing Components" at room temperature (RT) for half an hour.
3) The slides were washed 3 times for 15 minutes.

4) The first antibody diluted in Tris-buffered saline (TBS, PH 7.4) was added onto the sample and the incubation time was listed in the above table. TBS was used instead of the first antibody as negative control.
5) The slides were washed 3 times for 15 minutes
6) The second antibody (Powervision Mono-AP-Goat anti Mouse Ig G) was added to the sample and incubated at RT for 1 hour.
7) The slides were washed 3 times for 15 minutes
8) The colour-developing solution was prepared as below.
   (1) Solution I was composed of 18ml Aminomethylpropandiol-buffer, 50ml 0.05mol Tris-buffer (PH 9.7), 0.6g NaCL (0.15mol) and 28mg Levamisole. The first two solutions were mixed and then they were added into the beaker with NaCL and Levamisole (stirring them to promote dissolution).
   (2) Solution II was composed of 35mg Naphthol-AS-Bi-Phosphat in 0.5ml DMF (Dimethylformamid) solution.
   (3) Solution III was composed of 8 drops Neufuchsinlsg (5%) and 20 drops Natriumnitritlsg (4%).
   (4) First solution III was mixed with solution I, and then they were mixed with solution II. pH was adjusted to 8.7 with 2N HCL.
   (5) The colour developing solution was passed through the filtering paper.
9) The colour was developed as below:
   (1) The slides were incubated in the above solution at RT for 20 min.
   (2) The colour developing solution was decanted and the slides were washed with running cold water.
   (3) The sample was counterstained with Mayer's Hematoxylin for 10 to 20 seconds
   (4) The slides were first washed with warm water, and then washed with cold water.
   (5) The slide was mounted with the coverslip, and one drop of glyceringelatine was added between the sample and the coverslip.

#### 2.3.6.2. Peroxidase-anti-peroxidase (PAP) detection system

AFP and alpha1-antitrypsin were stained with PAP detection system (with the help of Frau Ute Möser, Frau Michaela Kasimir and Frau Renate Kern).

**Procedure of staining**

1) The sample was incubated with peroxidase-blocking reagent (DAKO, Denmark) for 5 minutes.

2) The slides were washed with "DAKO wash buffer" for 5 minutes. (The below wash step was the same as this).
3) The samples were incubated with normal pig serum (1:20) for 15 minutes.
4) The slides were washed.
5) The sample was incubated with the first antibody. TBS was used instead of the first antibody as the negative control. The incubation time was listed in above table.
6) The slides were washed.
7) The sample was incubated with "goat anti-rabbit immunoglobin" (1:40) for 30 minutes at room temperature.
8) The slides were washed.
9) The sample was incubated with PAP-complex (1:100) at room temperature for 30 minutes.
10) The slides were washed.
11) Preparation of colour-developing solution: 25mg 3-amino-9 ethylcarbazole were diluted in 5ml Aceton and 5ml Dimethylsulfoxid solution and then 40ml distilled water and 0.2ml 3% $H_2O_2$ were added into them.
12) The colour was developed as below:
    (1) The colour-developing solution was filtrated.
    (2) The slides were incubated inside the solution for 10 minutes.
    (3) The slides were washed with cold water.
    (4) The sample was counterstained with Mayer's Hematoxylin for 15 seconds.
    (5) First the slides were washed with warm water for 3 times, second with cold water for 3 times.
    (6) The slide was mounted with the coverslip, and one drop of Glyceringelatine was added between the sample and the coverslip.

### 2.3.7. Metabolism of lidocaine by human hepatocytes and intrahepatic BECs

In order to detect whether the proliferating BECs have activity of cytochrome P450 monooxygenase, lidocaine metabolism test was performed (with the help of Miss Mechthild Beste). The experiments were repeated 6 times in No.3 BECs line. Primary hepatocytes (On day 2 after culture) were checked at the same time.

**Process of lidocaine metabolism test on the BECs and hepatocytes**

1) Lidocaine was added into the culture medium to get an initial concentration of 5nM.
2) 1, 8, 24 hours later, 220µl supernatant were separately collected. Each time point was repeated 6 times.

3) The concentration of lidocaine in the supernatant was measured with TDx Analyzer machine (Fluorescence Polarization Immunoassay, FPIA).

### 2.3.8. Study of FCS, EGF, HGF, TGF-$\beta_1$ and human serum from liver resection patient on proliferation of the BECs. (MTT Method)

In order to analyze the growth regulation of BECs by growth factors, the effect of FCS (1 % and 10%), EGF (10 ng/ml), HGF (10 ng/ml) and TGF-$\beta_1$ (10 ng/ml) on the proliferation of BECs was studied. The concentrations of EGF and HGF were the same as in the growth medium, and 10 ng/ml TGF-$\beta_1$ was reported to be able to inhibit the proliferation of most epithelial cells in vitro. The human serum from the liver resection patient (Hepatocellular carcinoma patient) on the proliferation of the BECs was also checked.

During passage, the cells were plated in the 24-well plate coated with collagen G. Each well was plated 10.000 cells. The experiment was started one day after the plating. The cells were washed with PBS for 2 times, then the basic medium containing different factors were added.

The basic medium was Williams medium which contained 2mM glutamine, 0.02M HEPES, 5μg/ml insulin, 2μg/ml hydrocortisone, 40units/ml penicillin and 40μg/ml streptomycin. The human serum was taken just before the liver resection (day 0) and on day 1, 2, 3, 4 and 5 after operation. Each determination was performed 6 times.

**MTT**

1) After 24 hours, MTT method was used to detect the growth of the cells as below:
2) 110μl of MTT (4mg/ml) were added into each well (containing 1ml medium) in the 24-well plate.
3) Four hours later, the supernatant was removed, and 250μl of DMSO were added into each well to solubilize the formazan dye converted from MTT by the cells.
4) Five minutes later, the plate was shaken to make the solution homogenous. The dye was quantitated with an ELISA plate reader at wavelength of 550nm-690nm.

### 2.3.9. Statistical analysis

One-way ANOAVA with SPSS software (version 8.0) for windows was employed to compare the mean of different groups.

# 3. RESULTS

## 3.1. Isolation, culture and characterization of human hepatocytes

With the two steps of EGTA and collagenase perfusion methods, hepatocytes were stably isolated from eleven different liver tissues. The viability of freshly isolated hepatocytes was between 65-85% in these experiments. Freshly isolated hepatocytes are round and singly distributed. About 20 minutes after plating, hepatocytes adhered to the bottom of the culture plate coated with collagen G. Around 24 hours after plating, hepatocytes were confluent and presented typical polygonal shape ***(figue3.3)***. Hepatocytes were able to survive more than 2 to 4 weeks in culture.

Using FACS analysis, it was found that cultured hepatocytes expressed high level of CK 8, CK 18, ASGPR and low level of vimentin on the first day after plating ***(figure 3.1)***. Lidocaine metabolism experiments indicated they were capable of metabolizing lidocaine ***(figure 3.28)***. Indirect immunofluorescence staining identified that they were positive for CK 8, CK 18, albumin, ASGPR and alpha1 anti-trypsin ***(figure 3.4 and 3.5).***

## 3.2. Isolation and culture of human intrahepatic BECs

**Table 3.1 Summary of isolation and culture of intrahepatic BECs from 10 liver tissues**

| No. | Liver tissue | digestion time | Cell lines | passage times | proliferating time |
|---|---|---|---|---|---|
| 1 | 36 gram | 60/20 minutes | 1BECs | 12 | 18 weeks |
| 2 | 40 gram | 30/30 minutes | 2BECs | 11 | 12 weeks |
| 3 | 43 gram | 30/20 minutes | 3BECs | 10 | 12 weeks |
| 4 | 50 gram | 30/20 minutes | 4BECs | 7 | 7 weeks |
| 5* | 43 gram | 50/0 minutes | 5BECs | 3 | 4 weeks |
| 6* | 20 gram | 60/0 minutes | 6BECs | 3 | 4 weeks |
| 7 | 37 gram | 70/0 minutes | 7BECs | 3 | 4 weeks |
| 8 | 46 gram | 60/0 minutes | 8BECs | 3 | 4 weeks |
| 9 | 50 gram | 60/40 minutes | 9BECs | 3 | 4 weeks |
| 10# | 46 gram | 60/0 minutes | 9BECs | 0 | 1 week |

* The cells were freezed after the last passage.

# The cells were fixed at 1 week for experiment and stopped growing.

Isolation of human intrahepatic BECs included three main steps. The first step was to digest diced liver pieces with collagenase and to get liver cell suspension. The second step was to obtain nonparenchymal cells (NPCs) by differential density centrifugation. The third step was to purify BECs from NPCs with immunomagnetic separation method (anti-HEA). The above table summarized the results of isolation and culture of intrahepatic BECs from 10 different sources of liver tissues.

Freshly isolated BECs were round and formed clusters. Under contrast microscope, magnetic Dyna beads could be seen on the surface of BECs ***(picture3.6).*** From PBC and AIH liver tissues, the yield of BECs was very high, and the cells rapidly adhered and began to proliferate. About 12 hours after plating, many islands of BECs appeared ***(picture3.7).*** Therefore these cells were cultured with the growth medium (containing HGF) on the first day after plating. Some primary BECs were found still bound with the magnetic beads until they were passaged. The BECs from PBC and AIH liver tissue grew fast and became confluent in one $25cm^2$ flask in about 1 week after plating. However, after the passage, the growth speed of BECs decreased obviously.

On the other hand, the yield of BECs from children liver tissues (No. 1, 2, 3, 4) was lower, and it took about 72 hours for most of the BECs to adhere to the wall of culture flask. The BECs formed a few small islands. After the cells were cultured with growth medium containing growth factors, the BECs began to proliferate faster. On the average, the first passage of BECs from the children liver tissues was done on the $11^{th}$ day after culture. After passage, the BECs still proliferated as fast as before until 7 to 12 weeks after culture.

The viability of BECs after freezing and thawing was about 90%, and they kept the growth potential as usual and specific phenotype (see below).

### 3.3. Characterization of human intrahepatic BECs

#### 3.3.1. Morphology

Under phase contrast microscope, primary BECs were small and had oval shape. After passage, most BECs from PBC or AIH liver tissues became large and round, with many vacuoles in the cytoplasm. However, the BECs from children's liver tissues kept small and oval shape until they stopped proliferation ***(picture3.8).*** Moreover, hepatocyte-like BECs were found in the passaged BECs in this experiment, when some BECs became nearly confluent and the culture medium had been maintained for some time. These BECs had polygonal shape that was similar to hepatocytes', and they could survive for more than 4 weeks before the culture medium was changed ***(picture3.9).***

#### 3.3.2. FACS

Using FACS analysis, it was found that all isolated BECs in the culture expressed CK 7, CK 8, CK 18 and CK 19, while they did not express desmin. In primary BECs, the positive rate of vimentin was very low. However, in the proliferating and passaged BECs, the positive rate of vimentin increased. At the same time, most of the BECs were still positive for CK 19 ***(picture3.2, table 3.2).***

### 3.3.3. Indirect immunofluorescence staining

Indirect immunofluoresecence staining was in agreement with the results of FACS. All of the BECs in culture expressed CK 7, CK 8, CK 18, CK 19, while did not express desmin, factor VIII and ASGPR. HEA was positive in the primary BECs and negative in the late passaged BECs (after culture for 3 to 4 weeks). Vimentin expression was seen after BECs were passaged. ***(picture3.10-14)***. Albumin and α1-antitrypsin were negative in the primary BECs (No. 7 and No. 10 BECs lines were stained ) ***(picture3.15).*** However, they were positive after the BECs were passaged, and this included the BECs from the 1st to the 11th passage ***(picture3.18).*** CK 14 is believed to be the one of the liver progenitor cell markers present between 8 and 14 weeks' gestation during liver development. In this study, CK 14 positive BECs were not found *(table 3.2)*.

**Table3.2 Summary of FACS and indirect immunofluorescence analysis of the BECs**

| Antibodies | Primary BECs | Passaged BECs |
|---|---|---|
| HEA | + | - |
| CK 8 | + | + |
| CK 18 | + | + |
| CK 7 | + | + |
| CK 19 | + | + |
| CK 14 | - | - |
| Vimentin | - | + |
| Desmin | - | - |
| ASGPR | - | - |
| α1-antitrypsin | - | + |
| Albumin | - | + |
| Factor VIII | - | - |

### 3.3.4. Double-fluorescent immunostaining

In order to identify the cell origin of albumin and vimentin positive cells, double-fluorescent immunostaining was used in this experiment. The primary monoclonal antibodies of CK 7, CK 8 and CK 18 are all $IgG_1$ subclass, and FITC conjugated goat anti-mouse $IgG_1$ was used as their second antibodies. The positive cells for staining of these three antibodies present green colour. While the primary antibodies of albumin and vimentin are both $IgG_{2a}$ subclass, and TXRD conjugated goat anti-mouse $IgG_{2a}$ was used as their antibodies. The positive cells for staining of albumin or vimentin present red colour. The cells positive for both of $IgG_1$ subclass and $IgG_{2a}$ subclass present yellow colour under fluoresent microscope.

In the stained BECs (3BECs), most cells were both CK 7 and albumin positive, both CK 8 and albumin positive, both CK 7 and vimentin positive, and both CK 18 and vimentin positive ***(picture3.19-21).*** However, some BECs were only positive for CK 7 ***(picture3.19).*** This line of BECs was freezed on day 40 (the 3rd passage) after isolation and culture. The BECs were stained on day 4 after thawing.

### 3.3.5. Immunohistochemistry

Using immunohistochemistry technique, it was found that all stained BECs were strongly positive for Pan-CK, and this identified their epithelial origin ***(picture3.22).*** BECs from No. 2, 3, 4, 7 liver tissues were found to express AFP, c-kit, chromogranin-A and α1-antitrypsin, which are the markers of liver stem cells. According to the positive rate of the stained cells, the result was defined as – (negative), + (0-25% positive), ++ (25-50% positive), +++ (50-100% positive). The next table summarized the results of the immunohistochemistry.

**Table 3.3 The results of the immunohistochemistry of several BECs lines**

| Cell lines | Passage | AFP | c-kit | Chromogranin-A | α1-antitrypsin | Pan-CK |
|---|---|---|---|---|---|---|
| 2BECs | 10 | ++ | NE* | – | NE | NE |
| 3BECs | 2 and 5 | ++ | + | + | NE | +++ |
| 4BECs | 3 and 4 | ++ | + | + | + | +++ |
| 7BECs | 0 | ++ | ++ | NE | NE | NE |

*NE: Not Examined.

AFP expression was seen in all stained BECs, and in some cases most of the cells were strongly positive, including the 10th passage of 2BECs, the 3rd and 4th passage of 4BECs, and freshly isolated cells of 7BECs. It is worthwhile to point out that 4BECs line derived from a 10 years old donor normal liver tissue. C-kit, chromogranin-A and α1-antitrypsin were also positive in most of the stained BECs samples***(picture3.23-26)***.

### 3.3.6. RT-PCR

RT-PCR was used to detect the mRNA expression of albumin and AFP in No. 1, 2, 3, 4 cell lines. It was found that all these BECs lines expressed albumin mRNA (567bp) in the whole period of culture. AFP mRNA (531bp) was detected in the No. 2, 3, and 4 cell lines. It is also worthwhile to mention that the 9th passage of No. 2 BECs line was still positive for AFP mRNA. Primary hepatocytes were positive for albumin mRNA and negative for AFP mRNA. HepG2 cells were both positive for albumin mRNA and AFP mRNA. Raji cells were both negative for albumin mRNA and AFP mRNA in this experiment. Detection of GAPDH mRNA was served as the normalization ***(pictures3.27).***

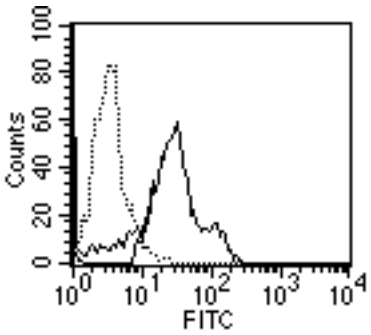
### 3.4. Metabolism of lidocaine by human hepatocytes and intrahepatic BECs

Six experiments were done to check the metabolism of lidocaine by BECs and hepatocytes. After the lidocaine was added into the culture medium, its concentration in the supernatant of the BECs kept unchanged with the time. While in the supernatant of hepatocytes, lidocaine concentration decreased obviously from 1 hour later ***(picture3.28).***

### 3.5. FCS, EGF, HGF, TGF-$\beta_1$, the serum of hepatectomy patient on the proliferation of BECs.

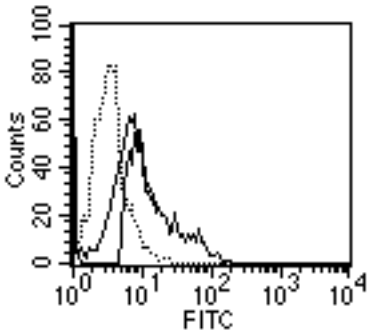
Using the MTT method to measure the growth of BECs, it was found that FCS (10 %), EGF (10 ng/ml) and HGF (10 ng/ml) separately promoted the proliferation of the BECs, while FCS (1 %) and TGF-$\beta_1$ (10 ng/ml) had no obvious effect on their proliferation ***(picture3.29).***

The human serum (5 %) from the 1$^{st}$ to the 5$^{th}$ day after liver resection enhanced significantly the proliferation rate of the BECs (P is less than 0.01 in all five time point), and this effect was most obvious in serum from the 2$^{nd}$ day after liver resection ***(picture3.30).***



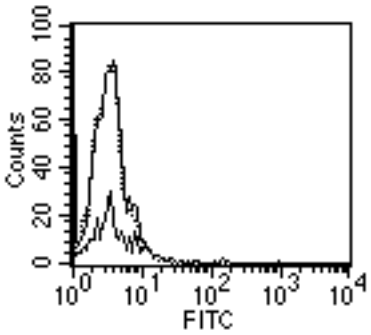

CK 8

| Events | % Gated | % Total | Mean | Median |
|---|---|---|---|---|
| 6574 | 82.17 | 38.33 | 38.54 | 28.13 |



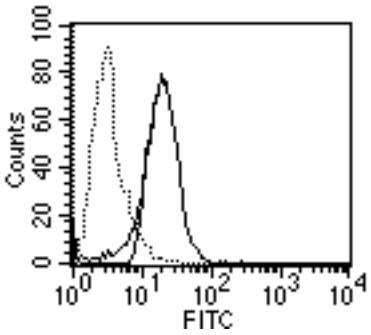

CK 18

| Events | % Gated | % Total | Mean | Median |
|---|---|---|---|---|
| 4917 | 61.46 | 33.21 | 17.56 | 10.55 |




Vimentin

| Events | % Gated | % Total | Mean | Median |
|---|---|---|---|---|
| 902 | 11.28 | 6.07 | 6.60 | 3.40 |




ASGPR

| Events | % Gated | % Total | Mean | Median |
|---|---|---|---|---|
| 7141 | 89.26 | 61.46 | 21.10 | 19.11 |

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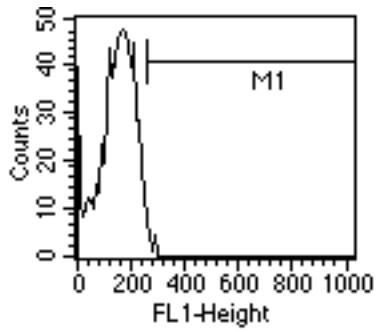
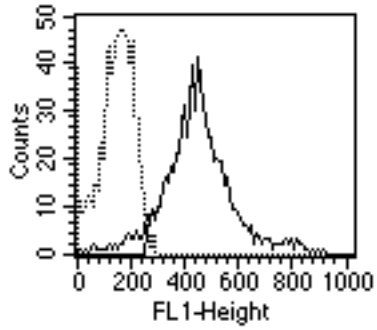
**Figure 3.1 FACS analysis of primary hepatocytes on day 1 after culture**
The expression of CK 8, CK 18, ASGPR was very high in the hepatocytes, while the expression of vimentin was very low.



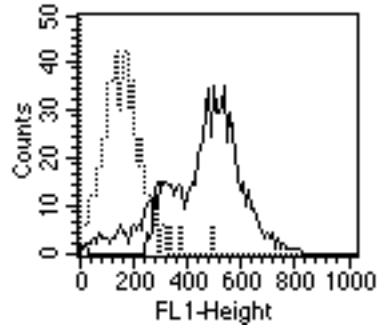

Negative

| Events | % Gated | % Total | Mean | Median |
|---|---|---|---|---|
| 5146 | 100.00 | 69.58 | 149.66 | 156.00 |
| 30 | 0.58 | 0.41 | 268.63 | 263.00 |




CK 19

| Events | % Gated | % Total | Mean | Median |
|---|---|---|---|---|
| 4906 | 95.63 | 62.93 | 445.85 | 437.00 |




Vimentin

| Events | % Gated | % Total | Mean | Median |
|---|---|---|---|---|
| 4816 | 86.67 | 52.86 | 476.02 | 486.00 |

**Figure 3.2 FACS analysis of BECs (No. 1) at the 6$^{th}$ week after culture**
The expression of CK 19 was still very high after the BECs were cultured for 6 weeks. However, the expression of vimenin was also high at this time.

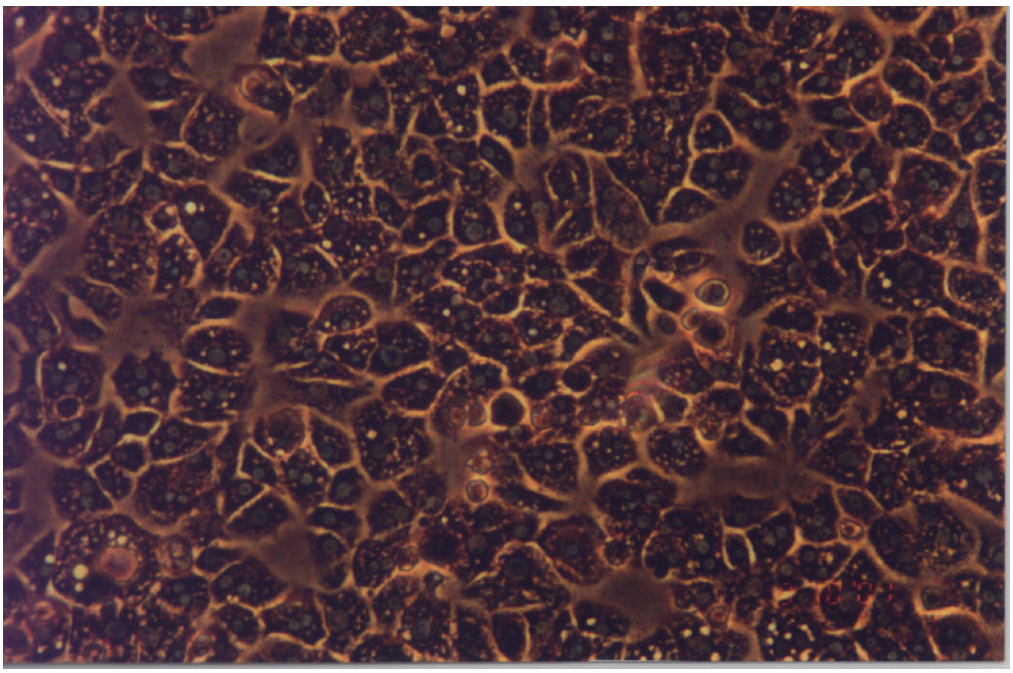


**Figure 3.3 Phase-contrast micrographs of primary hepatocytes on day 2 after culture (100 X)**
Hepatocytes were confluent and had typical polygonal shapes.

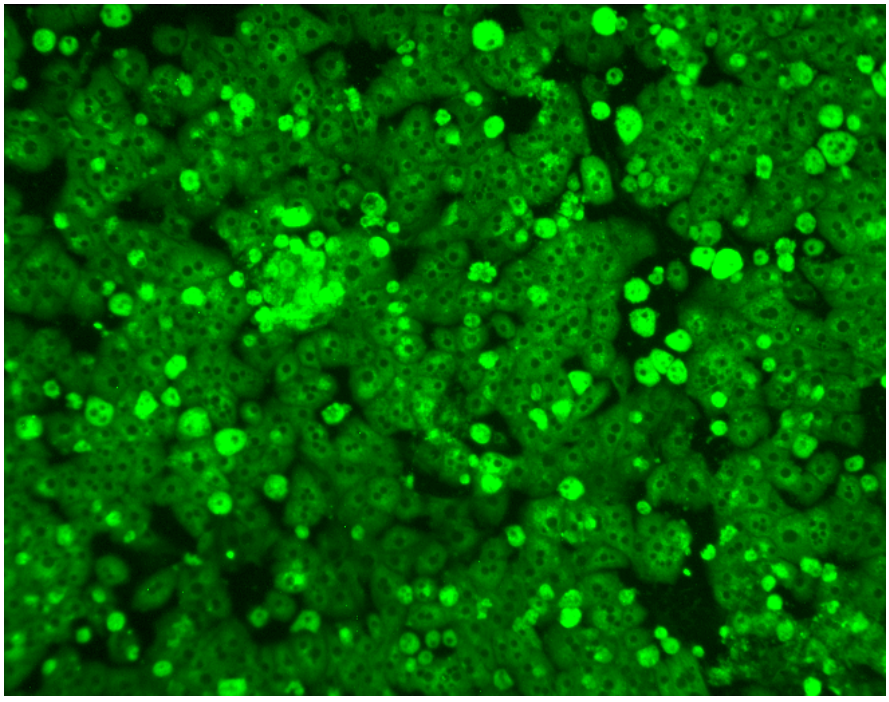


**Figure 3.4 Indirect immunofluorescent staining of primary hepatocytes with the alpha1-antitrypsin antibody**
The hepatocytes were stained on day 2 after culture (100X). FITC conjugated goat anti-mouse IgG was used as 2$^{nd}$ antibody. Positive cells presented green colour, and there were some non-specific stainings on the top of hepatocytes.

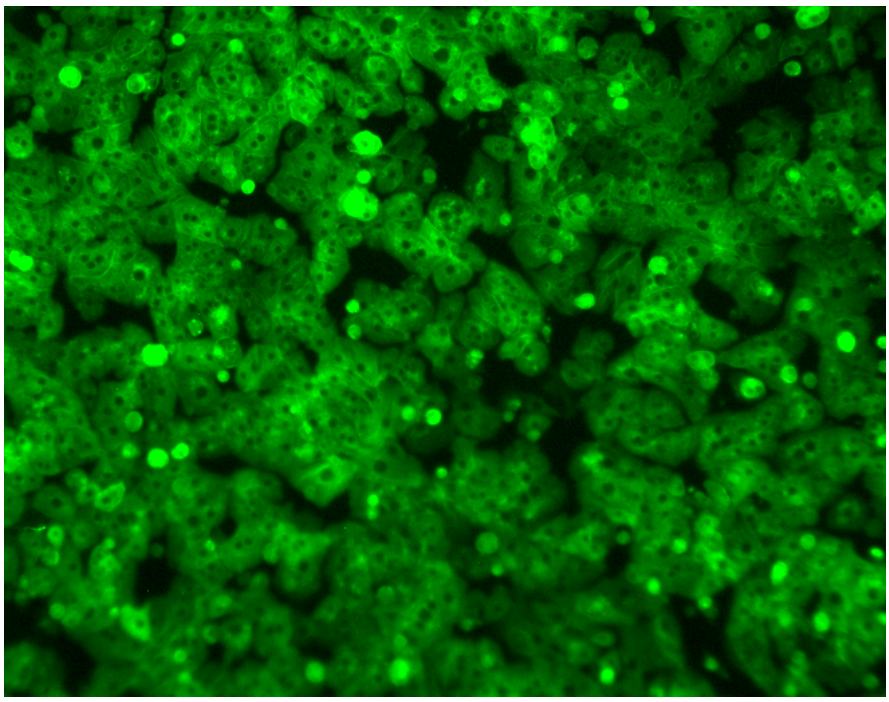


**Figure 3.5 Indirect immunofluorescent staining of primary hepatocytes with CK 8 antibody**
The hepatocytes were stained on day 2 after culture (100X). FITC conjugated goat anti-mouse IgG was used as 2nd antibody. Positive cells presented green colour, and there were some non-specific stainings on the top of hepatocytes.

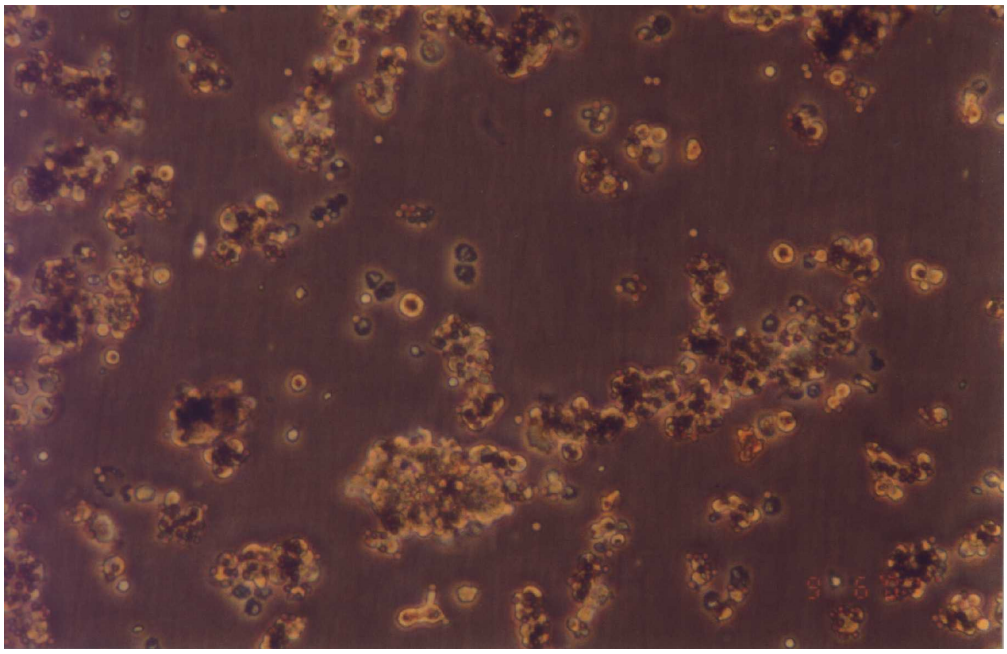


**Figure 3.6 Phase-contrast micrograph of freshly isolated BECs (100 X)**
BECs (No. 7) were white and round, forming clusters. Dyna beads were round and black, located on the surface of the cells.

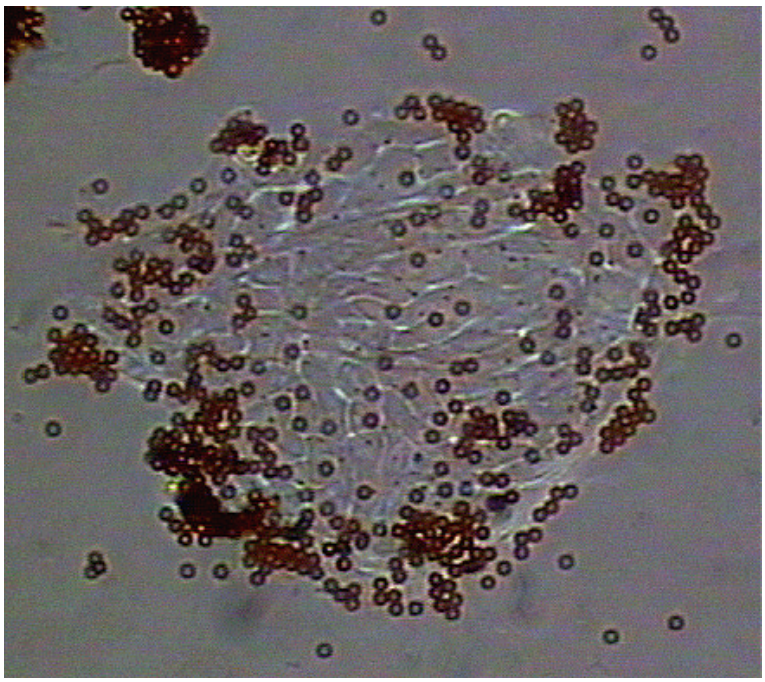


**Figure 3.7 Phase-contrast micrograph of BECs on day 1 after culture (100 X)**
BECs (No. 9) had adhered, proliferated and fromed an island. Dyna beads were on the surface of BECs.

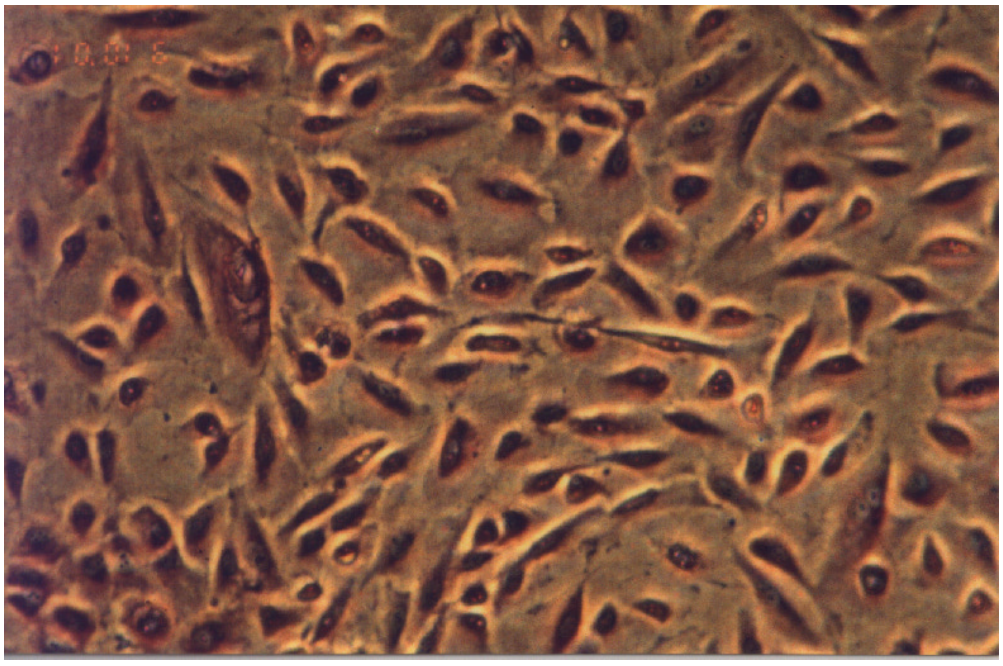


**Figure 3.8 phase-contrast micrograph of passaged BECs (100 X)**
The BECs (No.4) still had small and oval shape in the 3$^{th}$ week after culture.

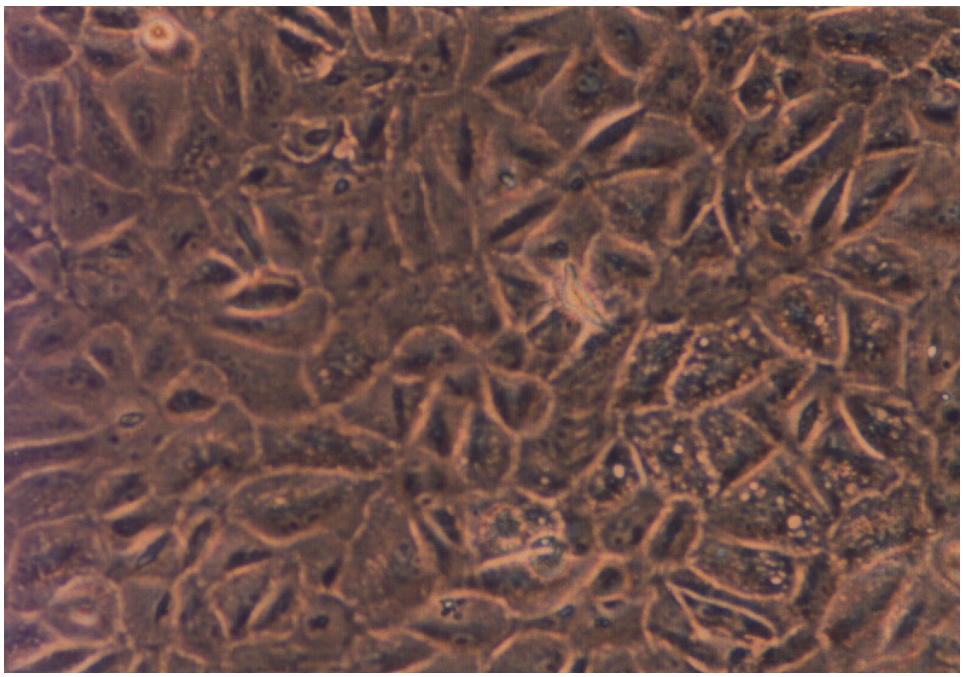


**Figure 3.9 phase-contrast micrograph of hepatocyte-like cells in passaged BECs (100 X)**
In the 5th week after culture, the BECs (No.4) were confluent and some had polygonal shape.

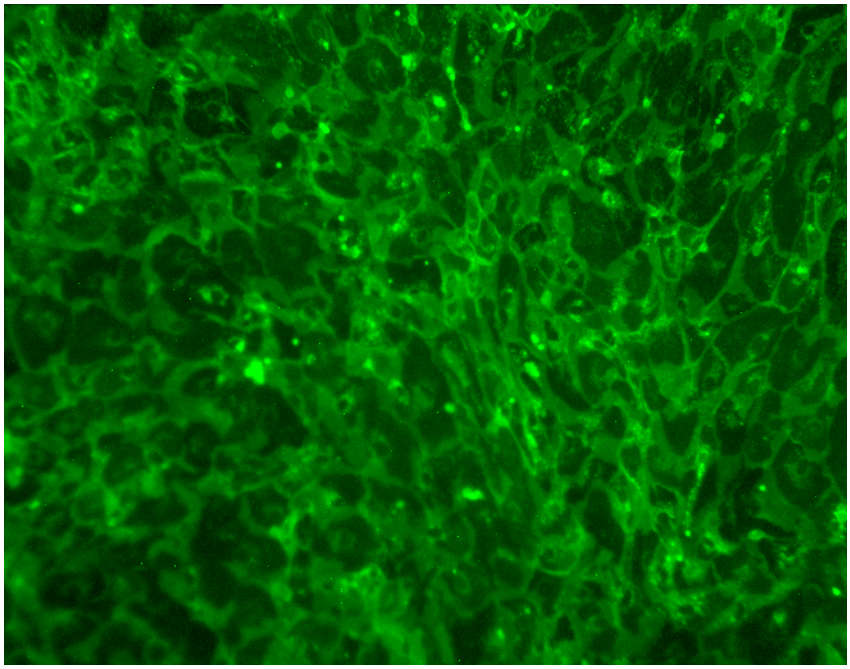


**Figure 3.10 Indirect immunofluorescent staining of BECs with anti-HEA (100 X)**
The BECs (No. 7) were stained on the 3rd day after culture. As HEA is located on the surface of the cells and the cell density was very high, the shape of BECs was not clear and the positive cells (green) formed net-like structure. FITC conjugated goat anti-mouse Ig G was used as the 2nd antibody.

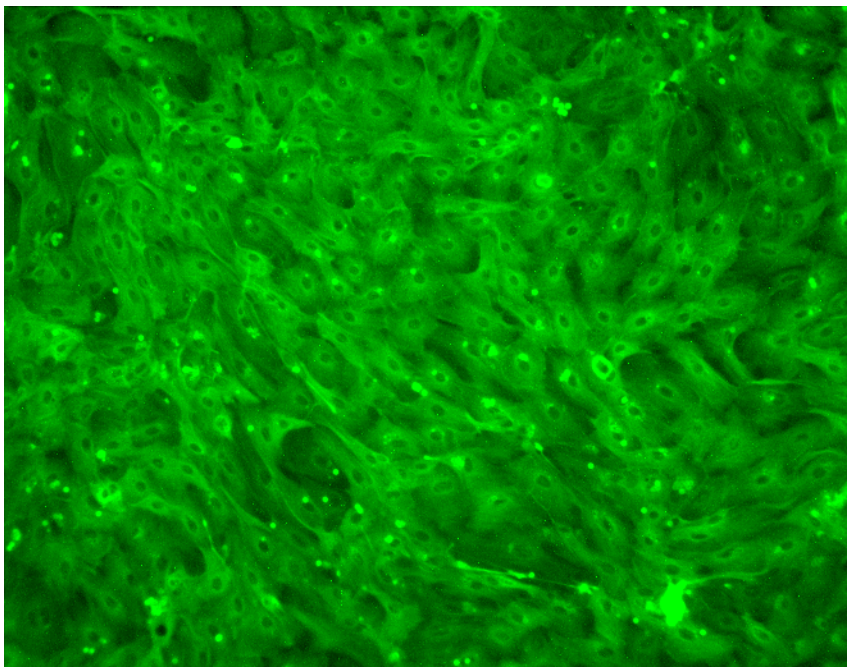


**Figure 3.11 Indirect immunofluorescent staining of BECs with CK 7 antibody (100 X)**
The BECs (No. 7) were stained on the 3rd day after culture. CK 7 is the specific marker of BECs. FITC conjugated goat anti-mouse IgG was used as the 2nd antibody. Positive cells presented green colour.

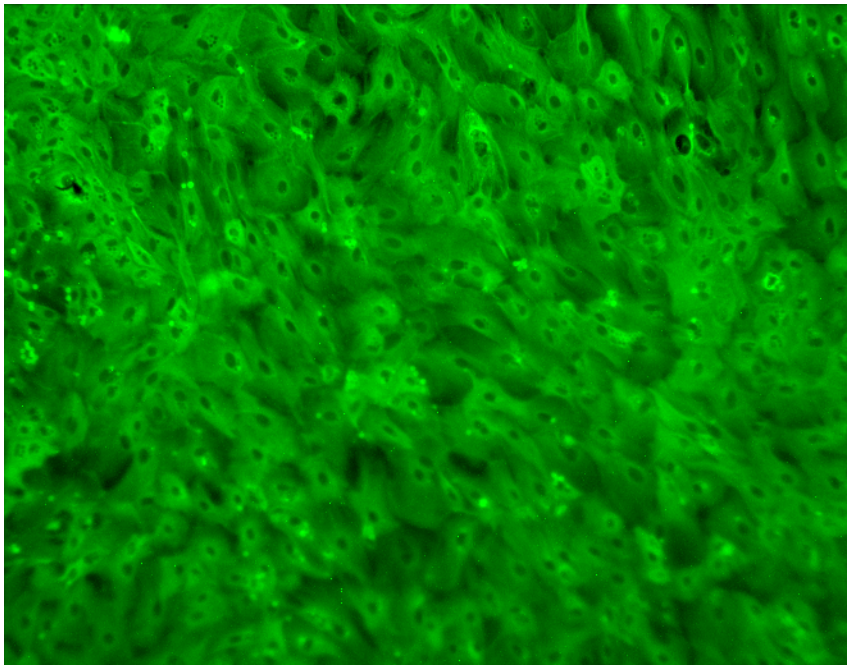


**Figure 3.12 Indirect immunofluorescent staining of BECs with CK 19 antibody (100 X)**
The BECs (No. 7) were stained on the 3rd day after culture. CK 19 is also the specific marker of BECs. FITC conjugated goat anti-mouse IgG was used as the 2nd antibody. Positive cells presented green colour.

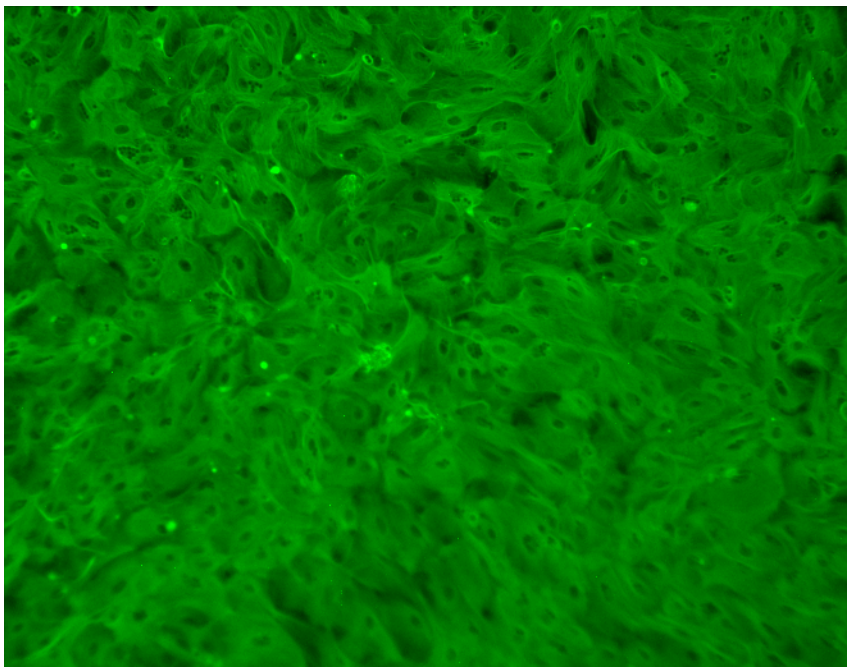


**Figure 3.13 Indirect immunofluorescent staining of BECs with CK 8 antibody (100 X)**
The BECs (No. 7) were stained on the 3rd day after culture. CK 8 is the specific marker of epithelial cells. FITC conjugated goat anti-mouse IgG was used as the 2nd antibody. Positive cells presented green colour.

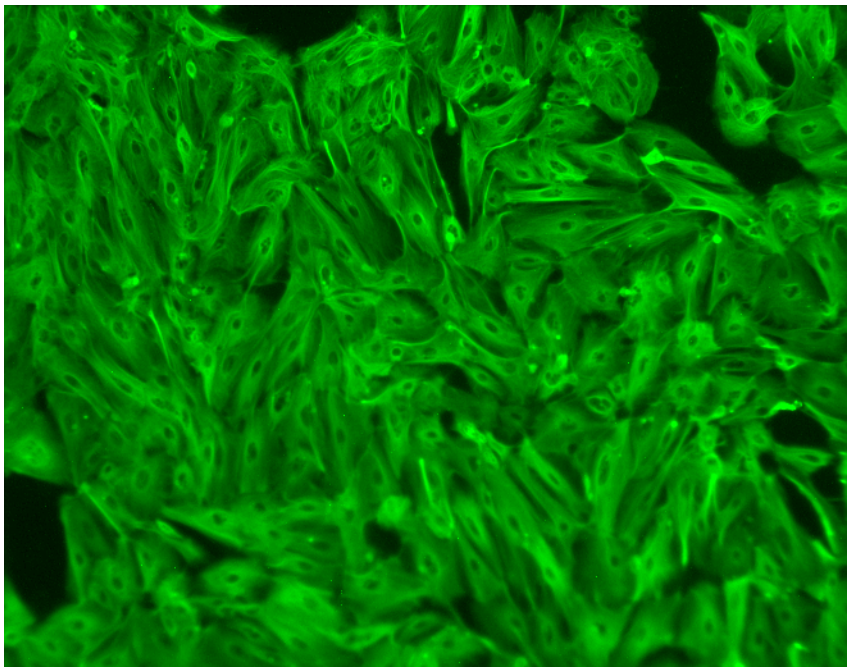


**Figure 3.14 Indirect immunofluorescent staining of BECs with CK 18 antibody (100 X)**
The BECs (No. 7) were stained on the 3rd day after culture. CK 18 is also the specific marker of epithelial cells. FITC conjugated goat anti-mouse IgG was used as the 2nd antibody. Positive cells presented green colour.

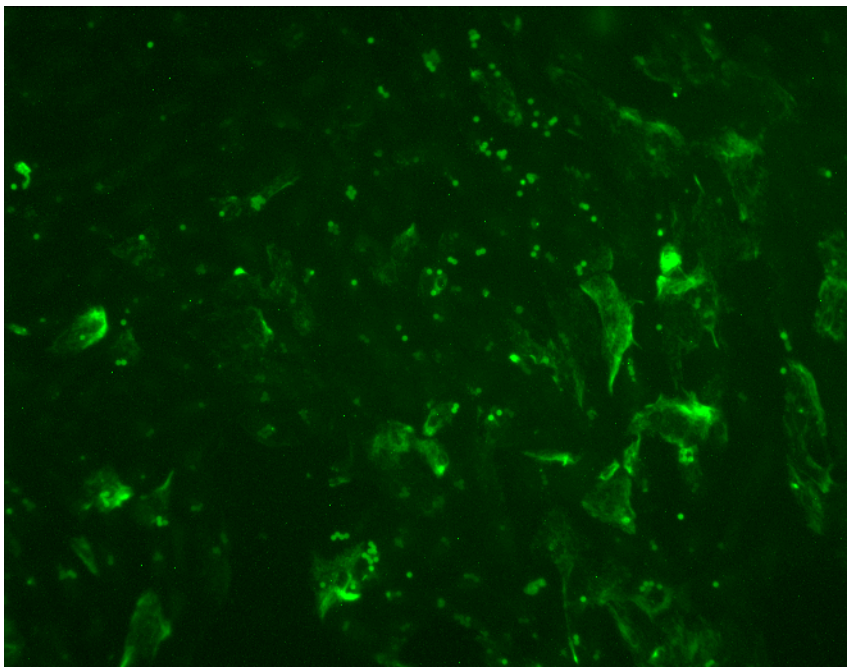


**Figure 3.15 Indirect immunofluorescent staining of BECs with vimentin antibody (100 X)**
The BECs (No. 7) were stained on the 3rd day after culture. Vimentin is negative in this primary BECs. Vimentin is the marker of mesenchymal cells. However, biliary cells in developing liver are positive for vimentin from 9 to 36 weeks of gestation. FITC conjugated goat anti-mouse IgG was used as the 2nd antibody.

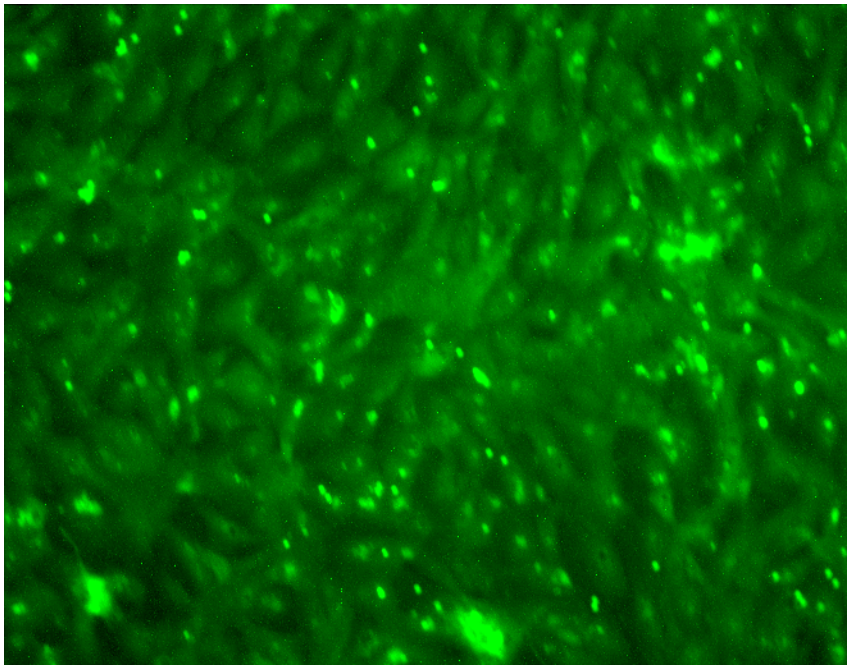


**Figure 3.16 Indirect immunofluorescent staining of BECs with albumin antibody (100 X)**
The BECs (No. 7) were stained on the 3rd day after culture. Albumin is negative in this primary BECs. Albumin is the maker of hepatocyte and liver stem cell. FITC conjugated goat anti-mouse IgG was used as the 2nd antibody.

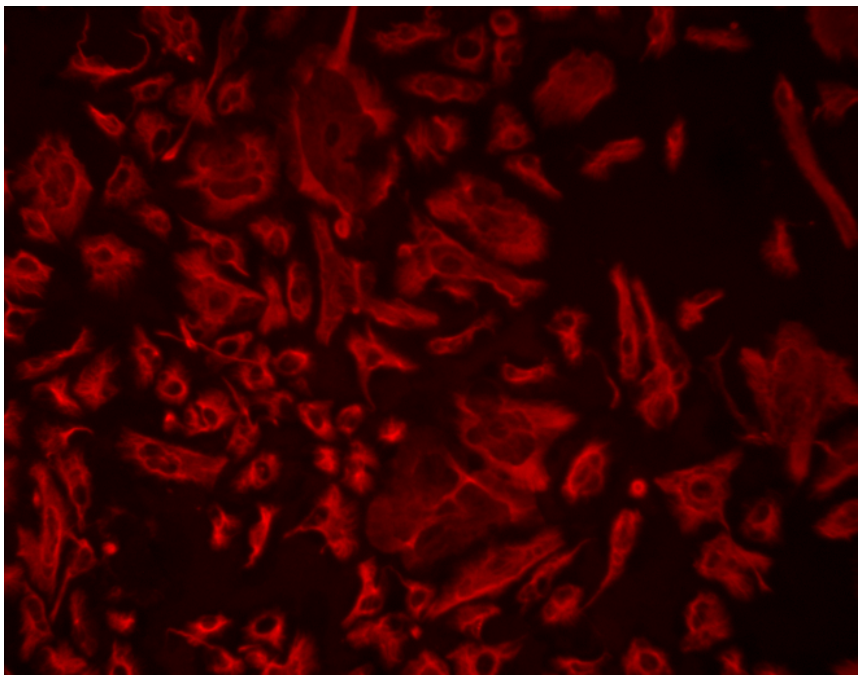


**Figure 3.17 Indirect immunofluorescent staining of BECs with vimentin antibody ($IgG_{2a}$) (100 X)**

The BECs (No. 3) were freezed on day 40 after culture, and they were stained on day 4 after thawing. TXRD conjugated goat anti-mouse $IgG_{2a}$ antibody was used as the 2nd antibody. Positive cells presented red colour.

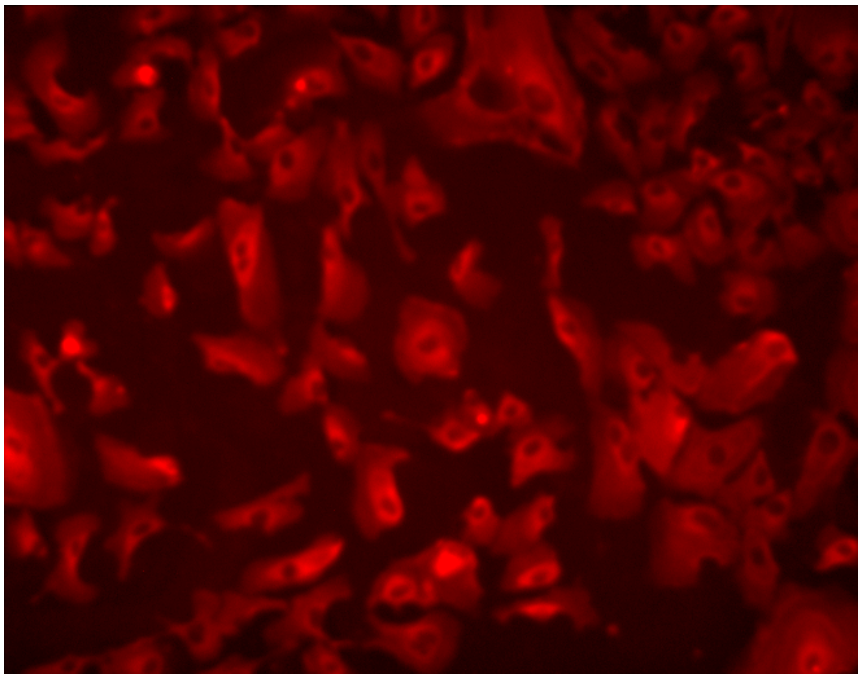


**Figure 3.18 Indirect immunofluorescent staining of BECs with albumin antibody ($IgG_{2a}$) (100 X)**

The BECs (No. 3) were freezed on day 40 after culture, and they were stained on day 4 after thawing. TXRD conjugated goat anti-mouse $IgG_{2a}$ antibody was used as the 2nd antibody. Positve cells presented red colour.

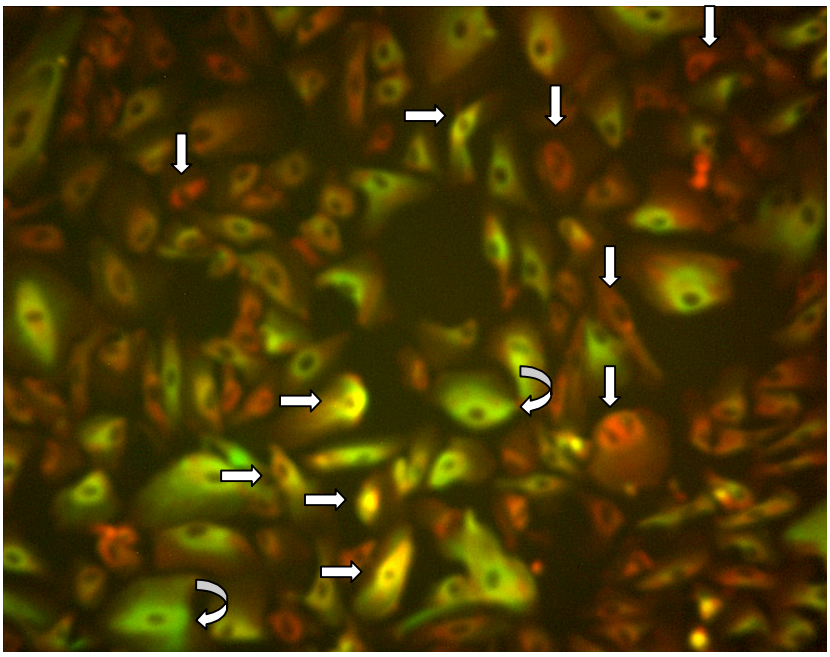


**Figure 3.19 Double immunofluoresent staining of BECs with CK 7 ($IgG_1$) and albumin ($IgG_{2a}$) antibodies**

The BECs (No. 3) were freezed on day 40 after culture, and they were stained on day 4 after thawing. FITC conjugated goat anti-mouse $IgG_1$ antibody was used as the 2nd antibody for CK 7 (green colour, curved arrow), and TXRD conjugated goat anti-mouse $IgG_{2a}$ antibody was used as the 2nd antibody for albumin ( red colour, vertical arrow). Both CK 7 and albumin positive cells present yellow colour (straight arrow)

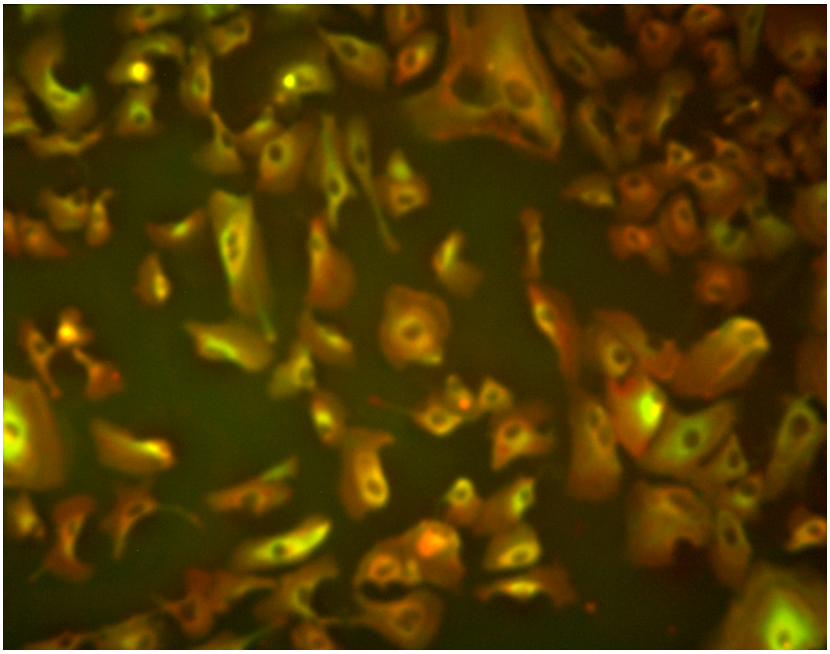


**Figure 3.20 Double immunofluoresent staining of BECs with CK 8 ($IgG_1$) and albumin ($IgG_{2a}$) antibodies**

The BECs (No. 3) were freezed on day 40 after culture, and they were stained on day 4 after thawing. FITC conjugated goat anti-mouse $IgG_1$ antibody was used as the 2nd antibody for CK 8 (green colour), and TXRD conjugated goat anti-mouse $IgG_{2a}$ antibody was used as the 2nd antibody for albumin (red colour). Both CK 8 and albumin positive cells present yellow colour. Most cells were both positive in this field.

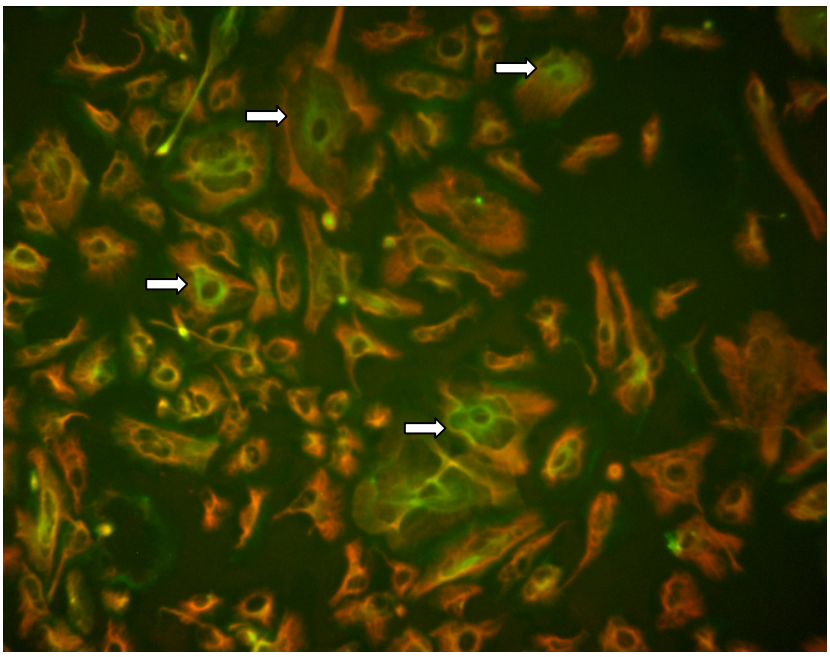
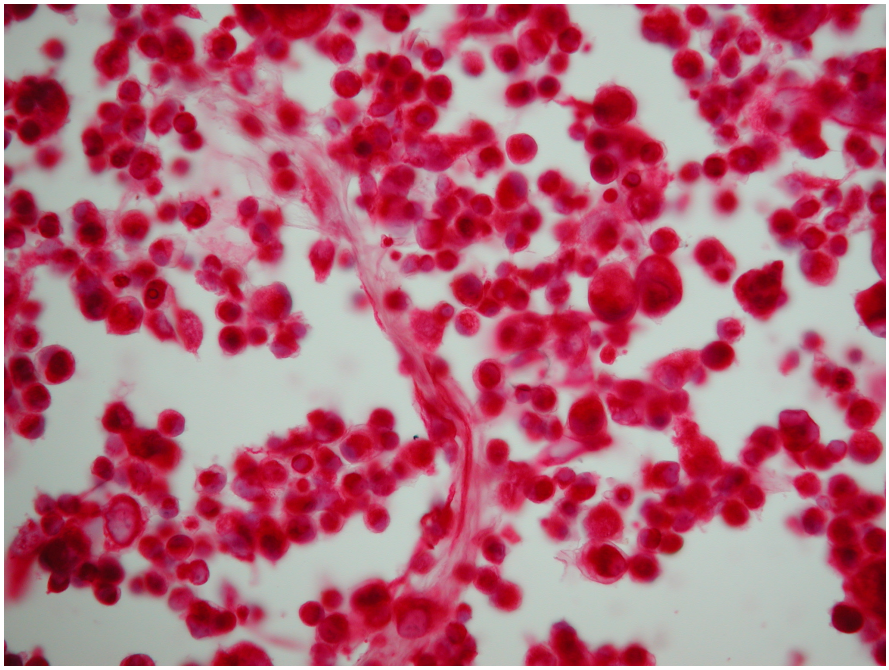
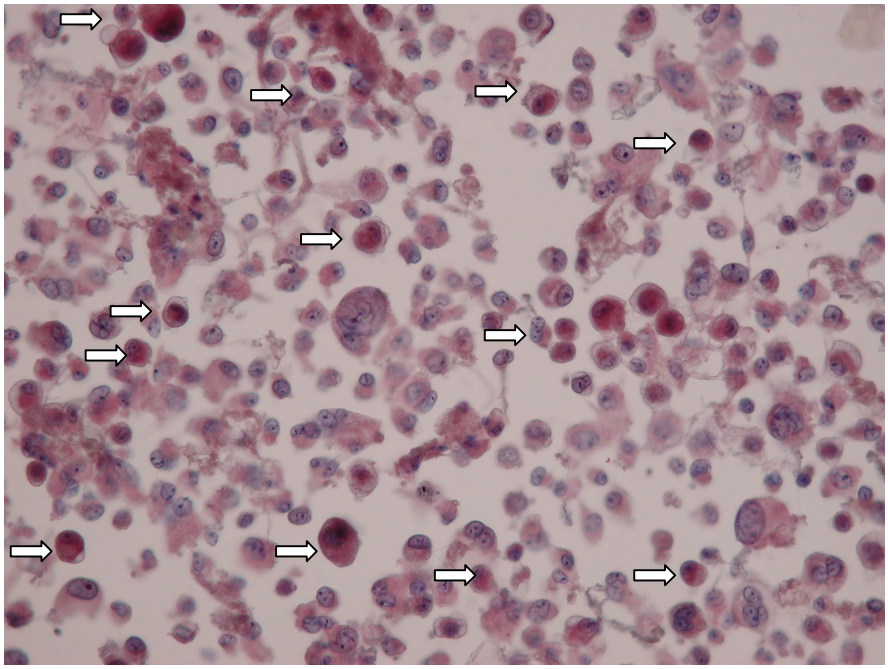


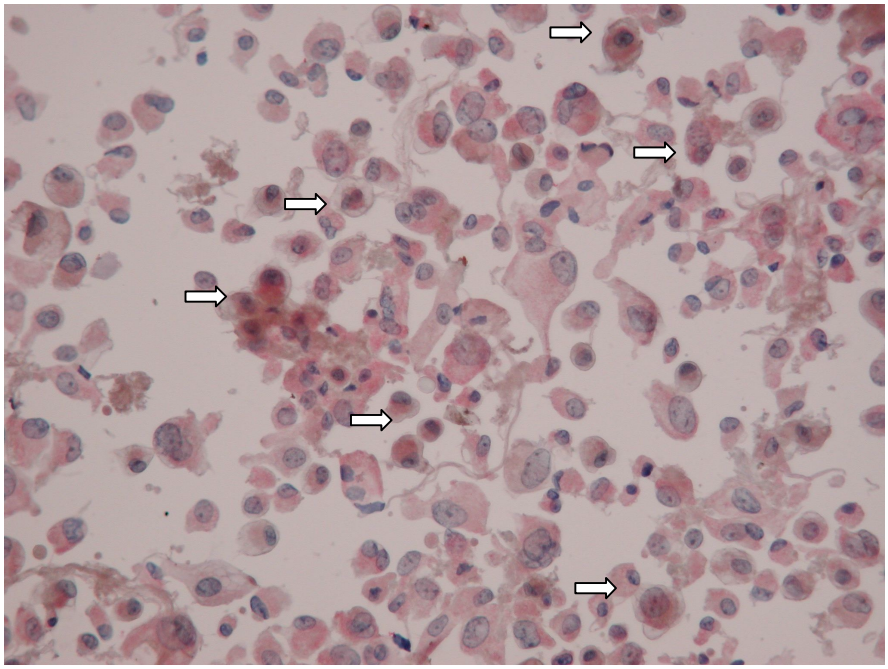
**Figure 3.21 Double immunofluorescent staining of BECs with CK 18 ($IgG_1$) and vimentin ($IgG_{2a}$) antibodies**
The BECs (No. 3) were stained on the 6th week after culture. FITC conjugated goat anti-mouse $IgG_1$ antibody was used as the 2nd antibody for CK18 (green colour, straight arrow), and TXRD conjugated goat anti-mouse $IgG_{2a}$ antibody was used as the 2nd antibody for vimentin ( red colour). Both CK 18 and vimentin positive cells present yellow colour. Most cells were both positive in this field.



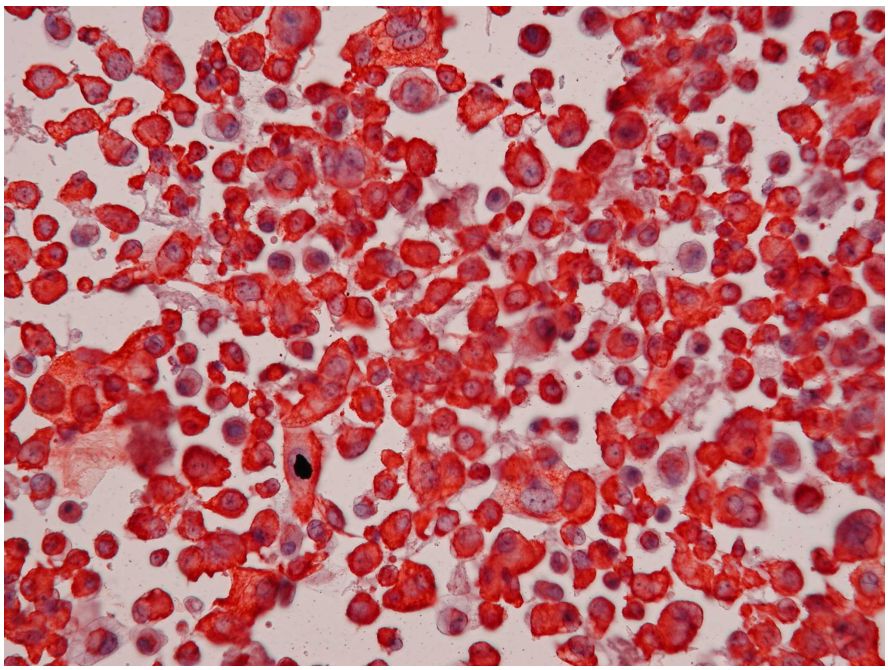
**Figue 3.22 Immunohistochemistrical staining of BECs with pan-CK antibody (100X)**
Pan-CK is the marker of epithelial cell. The BECs (No. 4) were stained in the 5th week after culture. All the cells are positive (red colour, PAP).



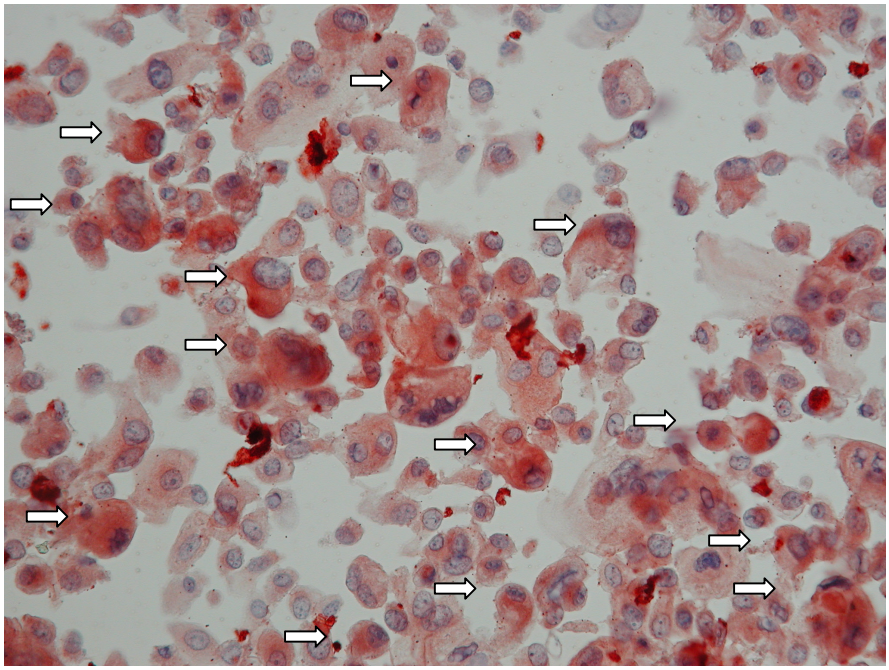
**Figue 3.23 Immunohistochemistrical staining of BECs with c-kit antibody (100X)**
c-kit is the receptor of stem cell factor and is expressed in ductal plate (primitive biliary cell) in developing liver. The expression of c-kit is also found in oval cells. The BECs (No. 4) were stained in the 5$^{th}$ week after culture. Some cells were positive. (red in cytoplasm, straight arrow; Powervision)



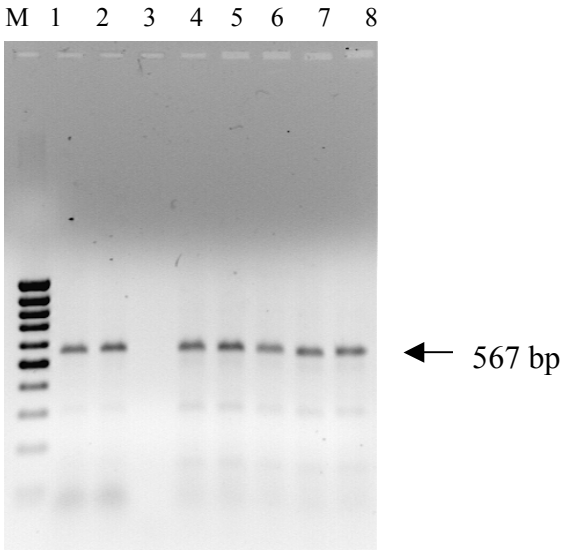
**Figue 3.24 Immunohistochemistrical staining of BECs with chromogranin-A antibody (100X)**
Chromogranin-A is expressed by reactive bile ductule and oval-like cells in diseased liver. The BECs (No. 4) were stained in the 5$^{th}$ week after culture. Some cells were positive (red in cytoplasm, Powervision).



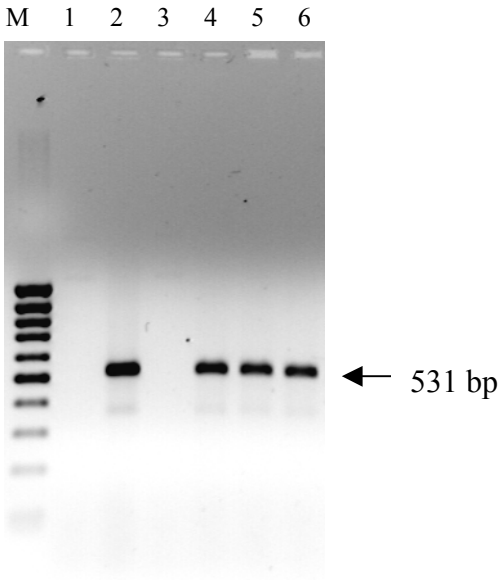
**Figue 3.25 Immunohistochemistrical staining of BECs with AFP antibody (100X)**
AFP is the marker of tumoral hepatocyte and liver stem cell. The BECs (No. 4) were stained in the 5$^{th}$ week after culture. Most cells in this field were positive (red colour, PAP). This BECs line was derived from a 10 years old donor liver tissue.



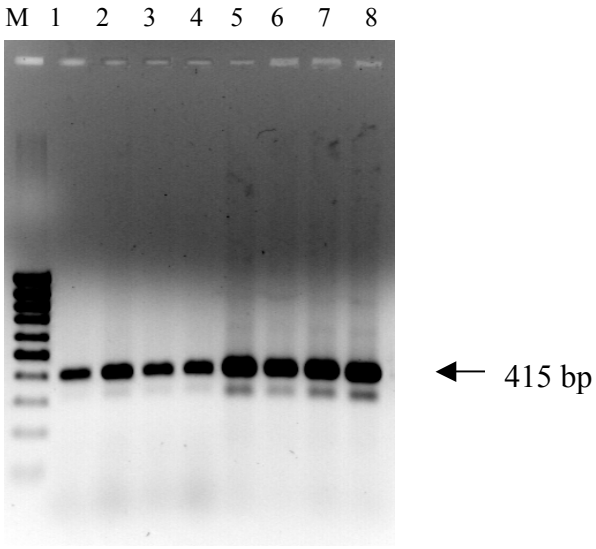
**Figue 3.26 Immunohistochemistrical staining of BECs with alpha1-antitrypsin antibody (100X)**
Alpha1-antitrypsin is the marker of hepatocyte and liver stem cell. The BECs (No. 3) were stained in the 7$^{th}$ week after culture. Some cells were positive (red colour, straight arrow; PAP).




Albumin RT-PCR products




AFP RT-PCR products




GAPDH RT-PCR products

**Figure 3.27 RT-PCR Products:**

M: DNA ladder; 1: primary hepatocytes; 2: Hep G2 cells; 3: Raji cells; 4: No. 3 BECs, in the 5$^{th}$ week after culture. 5: No. 4 BECs, in the 5$^{th}$ week after culture.6: No. 2 BECs, in the 8$^{th}$ week after culture. 7. No. 8 BECs, in the 1$^{st}$ week after culture. 8. No. 1 BECs, in the 6$^{th}$ week after culture.

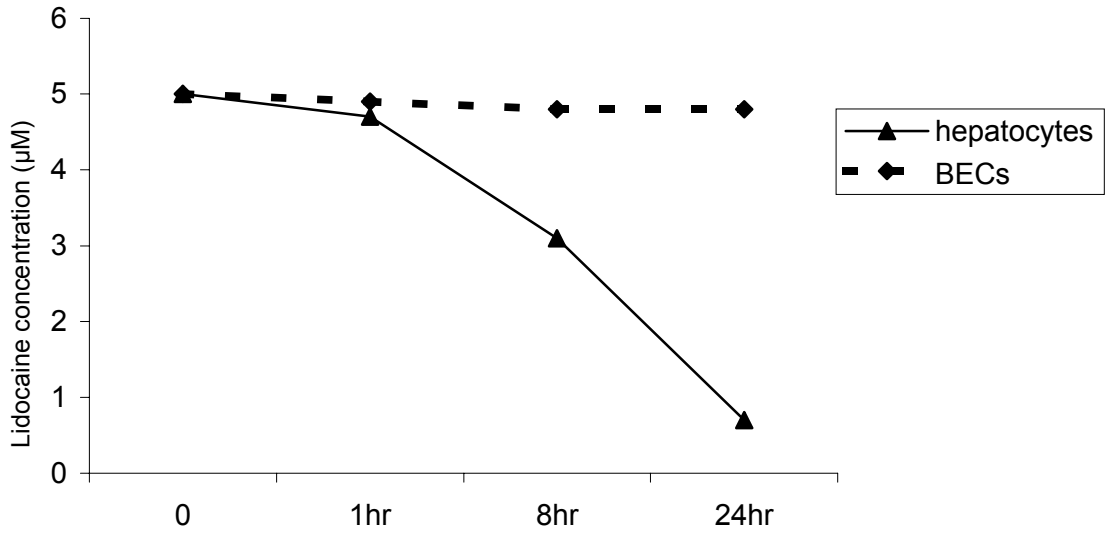



**Figure 3.28 The change of lidocaine concentration in the supernatant of the cells with time**
Lidocaine was added into the culture medium at the concentration of 5μM. One hour, 8 hours and 24 hours later, the supernatant was collected and the concentration of lidocaine was measured with fluorescence polarization immunoassay method. The concentration of lidocaine in the supernatant of hepatocytes decreased obviously from one hour after adding the drug, while it kept unchanged in the supernatant of BECs. The BECs were tested in the 7$^{th}$ week after culture. Each point represented the mean of six experiments.

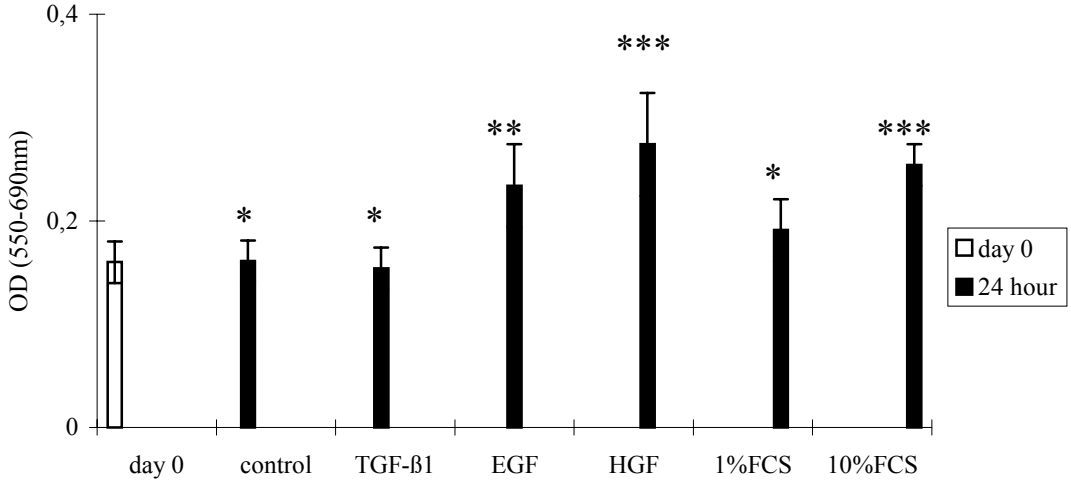



**Figure 3.29 The effect of growth factors and FCS on the proliferation of BECs**
The growth of BECs was measured with MTT method on day 0 and 24 hours after growth factors and FCS was added into basic medium. The value of each factor represented the mean of 6 to 8 experiments. Control (containing only basic medium), 10ng/ml TGF-ß1 and 1% FCS had no effect on BECs growth (* $P>0.05$). 10ng/ml EGF (** $0.01<P<0.05$), 10ng/ml HGF and 10% FCS (*** $P<0.01$) significantly promoted the growth of BECs. The BECs were tested in the 3$^{rd}$ week after culture.

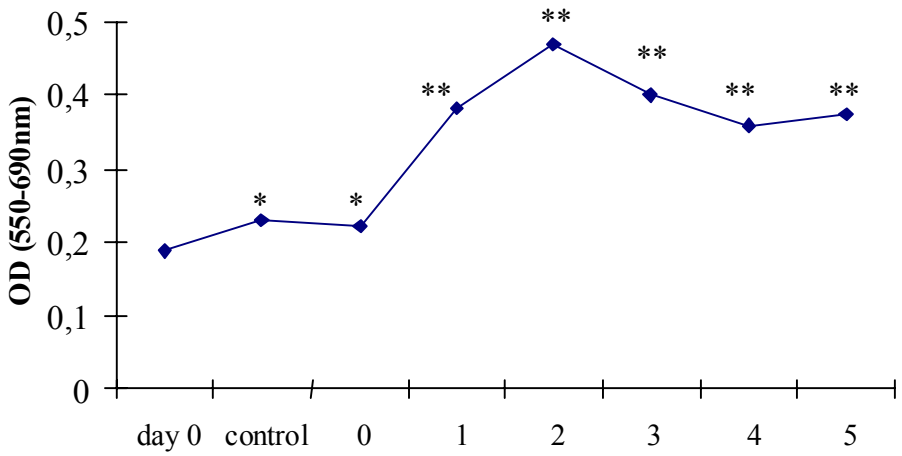



**Figure 3.30 The effect of human serum from hepatectomy patient on the proliferation of BECs**

The growth of BECs was measured with MTT method on day 0 and 48 hours after the serum (5%) was added into culture medium. The basic medium (control) and the serum taken before operation (0) had no effect on the growth of BECs. (* $P>0.05$), the serum taken after operation (day1-5: 1-5) significantly promoted the growth of BECs.(** $P<0.01$) Each value represented the mean of six experiments. The BECs were tested in the $7^{th}$ week after culture.

# 4. DISCUSSION

## 4.1. Isolation, culture and characterization of human hepatocytes

Human hepatocytes were isolated with EGTA and collagenase perfusion method in this study. The viability of freshly isolated human hepatocytes from different liver tissues was between 65% and 85%, and the primary hepatocytes could survive in the collagen G coated culture plate for 2 to 4 weeks. During this time, the hepatocytes presented the typical polygonal shape, and expressed epithelial cells markers, CK 8 and CK 18. They also expressed albumin, ASGPR, alpha1-antitrypsin and metabolized lidocaine, which are the characters of differentiated hepatocytes.

**Table 4.1 The markers detected in this experiment and their expressions in different liver cells**

| Marker | Hepatocytes | BECs | putative liver stem cells |
|---|---|---|---|
| ASGPR | + | - | - |
| CK 8 | + | + | + |
| CK 18 | + | + | + |
| CK 7 | - | + | + |
| CK 19 | - | + | + |
| CK 14 | - | - | + |
| c-kit | - | + (some) | + |
| Chromogranin-A | - | - | + |
| Albumin | + | - | + |
| AFP | - | - | + |
| Alpha1-antitrypsin | + | - | + |
| Vimentin | - | - | + |
| Cytochrome P450 | + | - | - |

## 4.2. Isolation, culture and characterization of human intrahepatic BECs

Human intrahepatic BECs were isolated with differential density centrifugation and immunomagnetic separation method from different liver tissues. BECs could proliferate in the culture medium (containing EGF and HGF) for at least 3 weeks, and some BECs proliferated for more than 18 weeks and were passaged for 12 times. Primary BECs were small and oval, and they expressed biliary cell markers (CK 7 and CK 19) and epithelial cell markers (CK 8 and CK 18), while they did not express desmin (marker of hepatic stellate cell), factor VIII (marker of endothelial cell), vimentin, alpha1-antitrypsin. All of these showed their biliary origin.

**However, in this study, our further characterization repeatedly found that proliferating BECs not only kept on expressing CK 7, CK 19, CK 8 and CK 18, but some also expressed c-kit, chromogranin-A, albumin, AFP, vimentin (after passage), alpha1-antitrypsin, which are the**

**markers of liver stem cells. Some of these BECs had the morphology of hepatocytes and could survive for more than 4 weeks without the change of medium. All of these phenomena suggested that there were some liver stem-like cells in intrahepatic BECs in vitro.**

### 4.2.1. Expression of c-kit and chromogranin-A by the BECs and its significance

c-kit is the receptor of stem cell factor (SCF). The stem cell factor and c-kit system is of critical importance for early epithelial stem cell differentiation in hematopoiesis or gametogenesis. Recent studies suggest that SCF and c-kit may also be involved in early growth and development of liver stem cells. Up-regulation of SCF and c-kit has been demonstrated in AAF-treated partially hepatectomized (AAF/PH) rats, in which AAF suppressed the replication of hepatocytes and the induction of oval cells was observed (Fujio et al. 1994). A similar increase of SCF and c-kit, accompanied by oval cell proliferation, has also been reported in bile duct-ligated rats (Omori et al. 1997). c-kit displays a peak expression in ductal plate cells in human liver until 22 weeks of gestation. In adult human liver, c-kit is detected in the Canal of Hering, which is the distal part of bile ductule, and possibly harbours liver stem cells (Theise et al. 1999). In severe human liver disease, such as fulminant hepatic failure, the expression of c-kit in the liver increased and some c-kit positive cells were also found integrated into bile ducts (Baumann et al. 1999). It was reported that isolated c-kit positive cells in human liver could differentiate into biliary cells in vitro (Crosby et al. 2001). In this experiment, some freshly isolated BECs and passsaged BECs were positive for the staining of c-kit, and this indicated that they might come from the Canal of Hering and possibly be related with an activation of liver stem cells in vitro.

Chromogranin-A was not detected in normal human liver. However, in liver regeneration after submassive necrosis and chronic cholestasis, putative progenitor cells in the vicinity of portal tracts, reactive bile ductule and hepatocyte-like cells were positive for the staining of chromogranin-A (Roskams et al. 1998). Thus, the expression of chromogranin-A by BECs in vitro could be the result of activation of putative liver stem cells.

### 4.2.2. Expression of AFP by the BECs and its significance

Using immunohistochemistry method, AFP expression was found in distinct BECs at different passages, including the BECs from normal donor liver tissue (No. 4 BECs). The expression of AFP mRNA was also identified in the BECs from No. 2 (at 8 weeks after culture), No. 3 (at 5 weeks after culture) and No. 4 (at 5 weeks after culture) liver tissues.

AFP is the major serum glycoprotein and carrier of polyunsaturated fatty acids during embryo-fetal development in vertebrates, and AFP synthesis is the characteristic of fetal cells. AFP appears to be one of the earliest markers of endodermal commitment toward liver cells, and AFP expression can

first be detected at 4 weeks of gestation in human. During the first weeks of biliary development from ductal plate cells (at about 7-9 weeks of gestation), the cells are also positive for AFP, which eventually becomes lost in developing bile duct cells (Haruna et al. 1996). Normal adult human liver do not express AFP. However, in normal rat liver, AFP mRNA was detected in situ hybridization in occasional nonparenchymal cells located in portal tracts, and these cells might be the progenitors of oval cells induced by some carcinogens (Alpini et al. 1992) (Lemire and Fausto 1991). Expression of AFP by human intrahepatic BECs in vitro strongly suggested the existence and activation of putative liver stem cells.

Additionally, freshly isolated BECs derived from AIH liver tissue (No. 7 BECs) were also positive for AFP. These positive cells could be ductular cells which were detected in chronic liver disease as reported and might be the result of activation of putative liver stem cells in vivo.

### 4.2.3. Expression of albumin and alpha1 antitrypsin by the BECs and its significance

Using RT-PCR technique, albumin mRNA (567bp) was positive in the BECs in each passage. However, with indirect immunofluorescence staining method, primary BECs were negative for the staining of albumin, while the passaged BECs (from the $1^{st}$ to the $11^{th}$ passage) become positive. Alpha1-antitrypsin was positive in the passaged BECs (the $5^{th}$ passage) with immunohistochemical staining.

It was reported that BECs purified with immunomagnetic separation method were contaminated by less than 1% hepatocytes (Joplin et al. 1990), and this could result the false positive of albumin mRNA in primary BECs. But in this experiment, albumin mRNA was also positive in the BECs from the $1^{st}$ to the $11^{th}$ passage. After the BECs were passaged for 11 times and survived in vitro for more than 4 months, the existence of contaminated hepatocytes was very unlikely. Moreover, the double-fluorescent immunostaining disclosed the co-expression of CK 7 and albumin, and this further supported that albumin positive cells were of biliary origin.

In human, albumin is expressed by both fetal and mature hepatocytes, and it is also expressed by ductal plate cells of fetal liver and ductular hepatocytes in diseased human liver. Bile duct cells and bile ductules are negative for albumin. Alpha1-antitrypsin has the similar expression pattern to albumin. Albumin mRNA was also detected in occasional nonparenchymal cells located in portal tracts in normal rat liver (Alpini et al. 1992). Thus, the expression of albumin and alpha1-antitrypsin by human intrahepatic BECs also indicated that there might be co-purified putative liver stem cells and they were activated in vitro.

### 4.2.4. Expression of vimentin by the BECs and its significance

Vimentin is considered as the marker of mesenchymal cells. However, in human developing liver, vimentin is detected in ductal plate and bile duct cells in the period between 9 and 36 weeks of gestation (Haruna et al. 1996). In normal human adult liver, only rare bile duct cells are positive for vimentin. Increased expression is associated with acute biliary obstruction and chronic liver disorders in human and oval cell proliferation in rat.

In this experiment, vimentin was negative in primary BECs, but positive in passaged BECs. Double-fluorescent immunostaining found the vimentin positive cells co-expressed CK 18 or CK 7, and this excluded the contamination by fibroblasts (mesenchymal cells). The expression of vimentin by proliferating human intrahepatic BECs again implied the activation of putative liver stem cells in vitro.

### 4.2.5. Lidocaine metabolism

Although some BECs in this experiment expressed albumin and alpha1-antitrypsin which are also the markers of hepatocytes, they could not metabolize lidocaine as hepatocytes. Lidocaine is metabolized by cytochrome P450 monooxygenase that exists only in hepatocytes. This result further excluded a major contamination with hepatocytes and indicated that albumin or alpha1-antitrypsin positive cells could be immature liver progenitor cells.

**The expression pattern of these BECs are similar to the bipotential liver progenitor cells in the human developing liver or oval-like cells in ductular reaction in some human diseased livers. This raised the next questions: What was the origin of the proliferating intrahepatic BECs in vitro? Did intrahepatic biliary tree consist of liver stem cells? How was liver stem cell activated in vitro?**

## 4.3. Origin of isolated HEA-positive human intrahepatic BECs

In this study, human intrahepatic BECs were purified with immunomagnetic (anti-HEA) separation method. HEA is a 34 KD surface glycoprotein expressed by many kinds of epithelial cells. However, in human liver, HEA was expressed only by biliary epithelium. Using immunohistochemistry technique, it was reported that large and medium sized bile duct, as well as bile ductule epithelium was stained positively for HEA antibody, but the hepatic cords themselves were completely negative (Joplin et al. 1989). Primary BECs isolated with immunomagnetic separation (anti-HEA) method were strong positive with staining of HEA in this experiment, and this identified their biliary origin.

The bile ductule is the smallest branch of the bile duct system. The Canal of Hering is the terminal portion of the bile ductule and it connects with the bile canaliculus which is formed by hepatocytes. The canal of Hering is composed on one side by the last hepatocyte and on the other side by the first biliary epithelial cell. It has been suggested that the Canal of Hering consists of, or harbours facultative hepatic stem cells in human. In normal human liver, the cells in the Canal of Hering express CK 19, c-kit, while do not stain for HepPar 1 and AFP (Theise et al. 1999). In this study, it was found that some BECs were positive with the staining of c-kit, and it indicated that these cells possibly came from the Canal of Hering.

Further, we found that some proliferating intrahepatic BECs also expressed albumin, AFP, alpha-1 antitrypsin and chromogranin-A which are the characters of liver stem cells. With double-fluorescent immunostaining, the cells, which co-expressed CK 7 and albumin, CK 8 and albumin, CK 7 and vimentin, CK 18 and vimentin were identified. These cells may derive from liver stem cells that existed in the intrahepatic BECs. In other words, some of the intrahepatic BECs may have the potential of liver stem cells. However, some CK 7-expressing BECs did not express albumin, and this showed that they were mature biliary cells which possibly came direct from medium and large branches of bile duct, or from the biliary differentiation of liver stem cells.

Compared with mature cells, stem cells have a greater potential to survive and proliferate. In this experiment, it was found that the yield of intrahepatic BECs isolated from end-staged diseased PBC and AIH liver tissues was very high, and the primary cells were confluent in about 5 to 7 days after culture. In these diseased liver tissues there were proliferation of bile ducts. While for the primary BECs isolated from children liver tissues, they were confluent in about 11 days after culture. However, BECs derived from PBC or AIH had a short proliferation period (4 weeks on the average). Although the yield of the BECs isolated from children liver tissues was much lower that that from PBC or AIH liver tissues, they had a longer proliferating time (7 to 12 weeks). This might imply that mature BECs took most part in the BECs isolated from PBC and AIH liver tissues, while BECs derived from child liver tissue might contain more cells with liver stem cell potential.

Thus, the isolated intrahepatic BECs may contain biliary cells in various differentiating stages. Bile ductule may contain liver stem cells and their early differentiating progeny, and these cells have a great potential to proliferate *in vitro*.

There are two convincing evidences suggesting that intrahepatic biliary tree in rodent liver may contain liver stem cell. Oval cells in rodent liver are believed to be progeny of liver stem cells, and they arise in the periportal area and form ductlike structure. The first evidence is that with the cholangiogram, the continuity between this ductlike structure and the existing portal bile ducts have been found (Dunsford et al. 1985) (Lenzi et al. 1992). The second is that bile ductular damage by toxin (4,4‘-diaminodiphenylmethane, DAPM) inhibits oval cell activation (Petersen et al. 1997).

### 4.4. Possible mechanism of activation of liver stem cells in vitro: role of growth factors

In this experiment, it was found that freshly isolated BECs or BECs in primary culture did not express albumin (at protein level by indirect immunofluorescent staining) and vimentin, while some cells acquired the expression of these two proteins and other liver stem cells markers during long term cell culture. Therefore, BECs with liver stem cell potential might be activated in vitro, and this might be related with the growth factors (EGF, HGF) in culture medium.

TGF-α and HGF are two important growth-promoting factors in normal liver regeneration. TGF-α is an autocrine growth factor, which is produced by hepatocytes and acts on it through binding the EGF receptor. HGF is produced by non-parenchymal cells (hepatic stellate cells) and stimulates hepatocyte replication by a paracrine or endocrine mechanism. HGF is the most potent known mitogen for hepatocytes *in vitro*. In the model of oval cell activation (AAF/PH), the first cells entering DNA synthesis are OV-6 and desmin positive cells in the periportal area and in the Glisson capsule. Coincident with the appearance of these cells, an increase in the expression of TGF-α and HGF was observed (Evarts et al. 1993). And this suggested that TGF-α and HGF were closely associated with the early activation of the liver stem cell compartment. EGF has the similar function to TGF-α, and they share the same receptor. *In vivo* infusion of EGF and HGF enhanced the mitogenic response of rat oval cells while decreased their apoptosis (Nagy et al. 1996). This further indicated that EGF and HGF were also essential to maintain the survival of oval cells. In human, oval-like cells were found in fulminant hepatic failure patient who had very high concentration of HGF both in peripheral blood and in liver tissue, and there might be relation between HGF and activation of liver stem cell.

In this experimental part, it was demonstrated that EGF (10ng/ml) or HGF (10ng/ml) significantly promoted the growth of BECs, and TGF-ß1 (10ng/ml) had no effect on their growth. The serum from the post-hepatectomy patient also stimulated the proliferation of BECs, while serum from the same patient taken before operation had no such effect. This can be explained by the increase of growth factors (TGF-α and HGF) concentration during liver regeneration. TGF-ß1 has antiproliferative effect on hepatocytes and stimulate hepatic stellate cells to produce extracellular matrix protein. TGF-ß1 is produced by hepatic stellate cells and acts in an autocrine/paracrine fashion (Fausto, 2000). As BECs did not grow in the basic medium in this experiment, the anti-proliferative effect of TGF-ß1 could not be found.

In this study, EGF and HGF not only could promote the proliferation of BECs *in vitro*, but might also activate the co-purified liver stem cells as they do *in vivo*.

### 4.5. Conclusion and prospective

From both normal and diseased human liver tissue, intrahepatic BECs were successfully isolated and cultured for some time *in vitro*. The characterization of these BECs demonstrated their specific phenotypes, moreover, liver stem-like cells were found in these BECs *in vitro*. This result supported the hypothesis that human biliary tree might also contain liver stem cells.

The discovery of liver stem cell in adult liver can be also used to understand the mechanism of hepatocarcinogenesis and cholangiocarcinogenesis. Dedifferentiation of mature cell and maturation arrest of liver stem cell are two models to explain the cell origin of primary liver (Sell1993) (Sell and Dunsford 1989). The discovery of liver stem cell in adult liver favours the maturation arrest theory. The liver stem cell may arrest in different differentiating stage, and transform into hepatoblastoma, hepatocellular carcinoma and cholangiocarcinoma. The further characterization of these primary liver tumors with liver stem cell markers may help to find their origin.

The liver stem cells have a great potential to proliferate. If they could be induced to differentiate into mature hepatocytes, there would be wide usage for hepatocyte transplantation and gene therapy of liver diseases.

However, based on this study, it is not clear how many putative liver stem cells were co-purified with the BECs, and how they were activated *in vitro*. Although the later is considered to be the action of growth factors (EGF and HGF), the exact dosage, duration and combination for the growth factors to activate liver stem cell need further investigation.

Based on this experiment, the location of putative liver stem cell in biliary tree also could not be identified. Some BECs were positive for the staining of c-kit, and this indicated they might come from the Canal of Hering. But the expression of c-kit might also be the result of activation of liver stem cell *in vitro*.

The putative liver stem cell in adult liver has been thought to be the remnant from developing liver in embryo. However, in past three years, there have been growing reports that bone marrow cells are capable of differentiate into hepatocytes in mice, rat and human (Theise et al. 2000b) (Alison et al. 2000; Lagasse et al. 2000; Theise et al. 2000a). This means that bone marrow cells may also be liver stem cells. Further studies to explore the proliferation and differentiation mechanism of liver stem cells hopefully will provide new insights, ultimately leading to better treatment of end-staged liver diseases.

# 5. ABBREVIATIONS

| | |
|---|---|
| AAF | 2-acetylaminofluorence |
| AAT | alpha1-antitrypsin |
| AFP | alpha-fetoprotein |
| ASGPR | asialoglycoprotein receptor |
| γ-GT | gamma glutamyl transferase |
| BECs | biliary epithelial cells |
| CK | cytokeratin |
| DAPM | 4,4‘-diaminodiphenylmethane |
| DHs | ductular hepatocytes |
| DMEM | dulbecco’s modified eagle medium |
| DMSO | dimethylsulfoxid |
| FACS | fluorescence activating cell sorting |
| FNH | focal nodular hyperplasia |
| HBSS | hanks’ balanced salt solution |
| HEA | human epithelial antigen |
| HepPar1 | hepatocyte parrafin 1 |
| HGF | hepatocyte growth factor |
| HSC | hepatic stellate cells |
| NPCs | nonparenchymal cells |
| RT-PCR | reverse transcription polymerase chain reaction |
| SCF | stem cell factor |

## 6. SUMMARY

Human hepatocytes were isolated with EGTA and collagenase perfusion method from eleven different liver tissues in this experiment. The viability of freshly isolated human hepatocytes was between 65% and 85%, and the primary hepatocytes could survive in the collagen G coated culture plate for 2 to 4 weeks. During this time, the hepatocytes presented the typical polygonal shape, and expressed epithelial cell markers, CK 8 and CK 18. They also expressed albumin, ASGPR, alpha1-antitrypsin and metabolized lidocaine, which are the characters of differentiated hepatocytes.

Human intrahepatic BECs were isolated with immunomagnetic separation method from ten different liver tissues. The BECs could proliferate in the culture medium (containing EGF and HGF) for at least 4 weeks, and some BECs had proliferated for more than 18 weeks and been passaged for 12 times. Primary BECs were small and oval, and they expressed biliary cell markers (CK 7 and CK 19) and epithelial cell markers (CK 8 and CK 18), while they did not express desmin (marker of hepatic stellate cell), factor VIII (marker of endothelial cell).

The further characterization repeatedly found that proliferating BECs not only kept on expressing CK 7, CK 19, CK 8 and CK 18, but some also expressed c-kit, chromogranin-A, albumin, AFP, vimentin (after passage), alpha1-antitrypsin, which are the markers of liver stem cells. Some BECs had the morphology of hepatocytes and could survive for more than 4 weeks without the change of culture medium. Double-fluorescent immunostaining identified the biliary origin of these cells. All of these phenomena suggested that there were some liver stem-like cells co-purified with intrahepatic BECs *in vitro*. Liver stem cells might be residents among intrahepatic BECs and activated in vitro by the growth factors.

This finding supported the hypothesis that the human biliary tree may harbour liver stem cells, and the liver stem cells could be co-purified with intrahepatic BECs.

# 7. LITERATURE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With a lot of thanks !

## 9. RESUME

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